7-23-2007

The Prevalence of Nelson Bay Virus in Humans and Bats and its Significance within the Framework of Conservation Medicine

Jennifer Betts Oliver

ABSTRACT

JENNIFER B. OLIVER

The Prevalence of Nelson Bay Virus in Humans and Bats and Its Significance within the Framework of Conservation Medicine

(Under the direction of KAREN GIESEKER, FACULTY MEMBER)

Public health professionals strive to understand how viruses are distributed in the environment, the factors that facilitate viral transmission, and the diversity of viral agents capable of infecting humans to characterize disease burdens and design effective disease intervention strategies. The public health discipline of conservation medicine supports this endeavor by encouraging researchers to identify previously unknown etiologic agents in wildlife and analyze the ecologic of basis of disease. Within this framework, this research reports the first examination of the prevalence in Southeast Asia of the orthoreovirus Nelson Bay virus in humans and in the *Pteropus* bat reservoir of the virus. Contact with *Pteropus* species bats places humans at risk for Nipah virus transmission, an important emerging infectious disease. This research furthermore explores the environmental determinants of Nelson Bay and Nipah viral prevalence in *Pteropus* bats and reports the characterization of two novel orthoreoviruses isolated from bat tissues collected in Bangladesh.

INDEX WORDS: Nelson Bay virus, Orthoreovirus, Nipah virus, Emerging infectious disease, Conservation medicine, Megachiropterans, Bats, Bangladesh

THE PREVALENCE OF NELSON BAY VIRUS IN HUMANS AND BATS AND ITS SIGNIFICANCE WITHIN THE FRAMEWORK OF CONSERVATION MEDICINE

By

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B.S. UNIVERSITY OF GEORGIA

A Thesis Submitted to the Graduate Faculty of Georgia State University in Partial

Fulfillment of the Requirements for the Degree

MASTER OF PUBLIC HEALTH

ATLANTA, GEORGIA

2007

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The Prevalence of Nelson Bay Virus in Humans and Bats and Its Significance within the Framework of Conservation Medicine

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ACKNOWLEDGEMENTS

In addition to my thesis committee and other faculty at the Institute of Public Health, I owe a debt of gratitude to the following people for their support and contribution to this work:

Pierre E. Rollin, MD, James A. Comer, PhD, MPH, and Thomas G. Ksiazek, PhD, DVM of the Special Pathogens Branch, National Center for Zoonotic, Vector-Borne and Enteric Diseases, Centers for Disease Control and Prevention for their inspiration, technical guidance, research expertise, and support of my educational endeavors during my employment. Thank you so much for granting me the time to research this project and to pursue this degree.

R. W. Compans of Emory University for donating a working stock of the Nelson Bay virus prototype strain.

Cynthia M. Goldsmith, MS of the Infectious Disease Pathology Branch, National Center for Zoonotic, Vector-Borne and Enteric Diseases, Centers for Disease Control and Prevention for the identification of unknown viral isolates and for training in electron microscopy techniques.

Darrin Carroll, PhD, MPH, Joel Montgomery, PhD, MPH, and the many other investigators who collected the specimens from Bangladesh I examined in this screening.

My husband, Brett W. Oliver, for his patience, tireless encouragement, and for making sure I didn't take all my meals out of vending machines.

My parents, John and Nancy Betts, for listening.

AUTHOR'S STATEMENT

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Carroll, D. S., Niezgoda, M., Montgomery, J. M., Kuzmin, I., Keeler, N., Gurley, E., Hossain, J., Azad, A. T., Mills, J., Ksiazek, T., Rollin, P., Comer, J. A., Oliver, J. B., Rupprecht, C., Akram, K., Croisier, A., Formenty, P., Bertherat, E., Breiman, R. F. (Publication in Preparation). Zoonotic Transmission of Nipah virus in Central Bangladesh.

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Chapter 1: Introduction

Background:

The U.S. Surgeon General infamously proclaimed in a speech to Congress in 1969 "it's time to close the book on infectious diseases." Recent generations of public health professionals have often marveled at this oversight while struggling to respond to a diverse range of new infectious agents and disease etiologies (Fauci, 2001). As summarized in a 2004 *Nature* Medicine Supplement article by Weiss and McMichael, the Institute of Medicine's landmark report in 1992 (IOM Report,1992) described diseases that are "caused by a newly evolving or newly occurring infection, established infectious diseases undergoing increased incidence or geographic spread, or newly discovered infectious agents causing a known infectious disease" as emerging infectious diseases (EIDs) (Weiss & McMichael, 2004, p. S70). First proposed by Taylor et al., many authors acknowledge that almost fifty percent of all human infections are due to diseases considered EIDs such as drug resistant tuberculosis and HIV/AIDS (Taylor et al., 2001). In addition, Feldmann et al. reiterated that societies around the globe are threatened by the at least 50 new diseases that have emerged in the past 20 years according to the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) (Feldmann et al., 2002; McMichael, 2004). EIDs comprise a significant threat to public health and as a result, interest in EID research has grown during the past decades (Lederberg et al., 1992; Newman et al., 2005).

It is a widely accepted view of EID researchers that few EIDs exclusively affect a single host; instead, most emerging diseases exist within a host continuum capable of infecting wildlife, domestic animals, and humans (Daszak et al., 2000, , 2001; Daszak et

al., 2004; Kruse et al., 2004). Approximately 75% of EIDs are of zoonotic origin and hence transmitted from animals to humans either directly or via an arthropod vector (Taylor et al., 2001). Therefore, the ecologic or environmental factors affecting either the zoonotic or human host play a particularly important role in the emergence or re-appearance of these diseases (Daszak et al., 2004; Feldmann et al., 2002; Kruse et al., 2004).

Cleaveland and colleagues recently categorized all known pathogens of humans, domestic livestock, and domestic carnivores based on the ability of the pathogen to jump species. They found that out of the 1,415 known human pathogens, a considerable 61.6% have a domestic animal origin (Cleaveland et al., 2001). It would be an enormous undertaking to add to this catalog a listing of wildlife pathogens capable of infecting humans in addition to the domestic animal sources examined. Moreover, since scientists estimate that only around one percent of the total number of human pathogens found in wildlife are known, such a listing would be woefully inadequate (Morse, 1993; Torres-Velez & Brown, 2004).

In the absence of vaccines, zoonotic disease control measures are largely directed at preventing zoonotic disease spread from animal or arthropod vectors to humans. However, characterizing the underlying determinants that affect disease prevalence in wildlife, performing surveillance in zoonotic disease hosts, and the identification of previously unknown infectious agents in wildlife are also critical components of zoonotic disease prevention (Daszak et al., 2004; Dobson, 2005; Kruse et al., 2004; Lynn et al., 2006; Steele, 1985; van der Poel et al., 2006). An increase in the prevalence of a virus in a zoonotic host reservoir may trigger a spillover event that results in human disease

(Calisher et al., 2006; Daszak et al., 2000). Although officials acknowledge that newly emerged novel zoonotic agents have often threatened public health, few studies have attempted to identify unknown agents in wildlife (Breed et al., 2005; Calisher et al., 2006; Daszak et al., 2004; Halpin et al., 2007; Steele, 1985; S. Wong et al., 2006). Specifically, the ecologic conditions that precipitate the transmission of pathogens harbored by mosquitoes and other insects, ticks, snails, rodents, and bats are repeatedly identified by scholars as one of the most important areas of human EID research (Morens et al., 2004; Zinsstag et al., 2007).

Although bats are most often incriminated in the transmission of rabies and other lyssaviruses, bats also play a lesser-known role in the transmission of such devastating human viral illnesses as SARS, Ebola and Marburg, West Nile, Kyasanur Forest disease, Japanese Encephalitis, Chikungunya, Venezuelan equine encephalitis, Hantaan, Rift Valley fever, and Influenza A (Breed et al., 2005; Calisher et al., 2006; Dobson, 2005; Halpin et al., 2007; van der Poel et al., 2006). Yet surprisingly, the full diversity of the bat viral landscape, sometimes referred to as bat viral ecology, remains undiscovered (Breed et al., 2005; Calisher et al., 2006; Halpin et al., 2007; S. Wong et al., 2006).

Nipah viral encephalitis is a newly recognized zoonotic EID which has caused outbreaks of severe encephalitis in humans throughout Southeast Asia since 1999 (Chua et al., 1999) and is transmitted to humans from bats either directly or via an animal intermediary (Field et al., 2001; Parashar et al., 2000; Yob et al., 2001). Infections caused by many of the bat-transmitted EIDs, such as Nipah virus encephalitis, are rare relative to the morbidity and mortality caused by other infectious disease threats to public health. Nevertheless, bat-transmitted EIDs remain threatening due to their high case

fatality ratios, lack of effective therapy, public dread, threat to the agricultural industry, and potential bioterrorism use (Daszak et al., 2004; Feldmann et al., 2002; Lederberg et al., 1992; McMichael, 2004; Newman et al., 2005).

Researchers have proposed that the frequency of Nipah virus outbreaks in Southeast Asia may be increasing due to environmental pressures, many of which are the result of human actions (Chua, Chua et al., 2002; Daszak et al., 2001; J. H. Epstein et al., 2006; Weiss & McMichael, 2004). Furthermore, some researchers believe that EID zoonoses often occur in unhealthy ecosystems since zoonotic EIDs have strong environmental determinants (Alcamo et al., 2003; Cook et al., 2004). These environmental determinants might likewise increase the prevalence of other viruses maintained in bat reservoirs not yet associated with human or wildlife disease (Daszak et al., 2001; van der Poel et al., 2006). An increase in the prevalence of a known viral agent in bats may provide an early warning indicator of the existence of favorable conditions that could likewise precipitate spillover of an unknown bat virus into humans. Therefore, monitoring the bat prevalence of viruses causing known disease in humans, like Nipah virus, may also serve as a surrogate measure of overall ecosystem health (Alcamo et al., 2003; Cook et al., 2004).

The prevalence of zoonotic diseases in human and animal hosts is commonly determined based on antibody evidence of viral infection by serology. Enzyme-linked immunosorbant assays (EIAs) have been developed to test for antibodies in bats and humans against EIDs such as SARS, Nipah, Hendra, and Ebola because they provide a quick and cost-effective way to determine exposure (Daniels et al., 2001). Previous research has demonstrated that Nelson Bay (NB) virus and other newly discovered

viruses from the orthoreovirus family are circulating in bats of the *Pteropus* genus in Southeast Asia and Australia. However, the prevalence of these viruses in their bat hosts remains unknown (Chua et al., 2001; Dixon, 2007; G. P. Gard & Marshall, 1973; Halpin et al., 2007; Pritchard et al., 2006; S. Wong et al., 2006). In fact, NB virus has been studied so little that fewer than a dozen papers have ever referenced it in the literature since it was first detected in 1970 (G. Gard & Compans, 1970). Although bat orthoreoviruses, like NB virus, are currently not suspected to be agents of human disease, screening for antibody evidence of NB virus has not been attempted. No diagnostic assay currently exists to detect the presence of NB virus. A serologic assay to detect antibody to NB virus may be a useful diagnostic tool if NB virus were to cross species barriers to cause disease in domestic animals, wildlife, or humans.

Research Rationale:

The environmental determinants affecting the prevalence of Nipah virus in bats from Southeast Asia might similarly be affecting the prevalence of NB virus since both viruses share a common reservoir, bats of the genus *Pteropus*. With a geographic distribution that spans Southeast Asia and Australia, some *Pteropus* bats share a high degree of contact in camps of overlapping species (Wilson & Reeder, 2005). If NB virus is shed in the bodily fluids of *Pteropus* bats, as has been documented for Nipah virus (Chua, Koh et al., 2002), it is possible that NB virus would be efficiently transmitted to other cohabitating bat species so that it is found throughout the geographic distribution of *Pteropus* bats.

Developing an EIA assay to detect NB viral antibody would allow researchers to explore the seroprevalence of NB virus. Determining the seroprevalence of NB virus in a bat sera catalog containing known antibody evidence of Nipah viral infection may elucidate temporal or geographic similarities in the prevalence patterns of both viruses. If pattern similarities are identified, they may help researchers better characterize the role that environmental determinants play in affecting the prevalence of bat viruses. To explore these hypotheses, a NB virus EIA was developed to screen a collection of over 2,323 rare bat sera specimens collected throughout Southeast Asia from 1993 through 2006. The bat sera specimens were previously determined to have antibody evidence of Nipah virus infection and were tested for NB viral antibody to better understand the distribution of this unexplored bat virus.

To further characterize the viral ecological spectrum in bats, which are important disease host, 168 bat liver and spleen tissue specimens associated with a 2004 Bangladeshi human Nipah virus outbreak from eight bat species were screened for the presence of virus by cell culture. Although previous studies have indicated that most novel viruses identified are unlikely to be of direct significance to human or animal health, novel nonpathogenic virus isolates may nevertheless help describe the full diversity of the viral ecologic spectrum in bat reservoirs of human disease (Calisher et al., 2006; Halpin et al., 2007).

The Southeast Asian human populations that experienced outbreaks of Nipah virus have unique environmental, lifestyle, and behavioral attributes that may have resulted in their exposure to other bat-transmitted viruses. It is unknown if the environmental, lifestyle, and behavioral risk factors present in the Southeast Asian

populations that experienced outbreaks of Nipah virus also exposed the populations to NB virus. Although bat orthoreoviruses, like NB virus, are currently not suspected to be agents of human disease, screening for antibody evidence of NB virus has not been attempted. Screening a Nipah virus outbreak associated human sera catalog previously tested for Nipah viral antibody may therefore lead to the identification of other bat viruses previously unknown in humans, like NB virus. Therefore, 1,861 human patient specimens collected during Nipah virus outbreaks in Southeast Asia from 2001 through 2006 and previously tested for Nipah viral antibody were also screened for NB virus using the newly developed EIA.

Traditional epidemiological research has focused on identifying risk factors specific to human disease. Larger ecologic trends influencing disease emergence on a global scale are often unrecognized (Farmer, 1996; Lederberg et al., 1992). The newly acknowledged public health discipline of conservation medicine encourages a macroscopic approach to disease study by examining the underlying ecologic causes of disease emergence using multidisciplinary techniques often considered outside the confines of the both the conventional analytic and social sciences (Daszak et al., 2001). Conservation medicine strives to examine the full diversity of pathogens in important wildlife reservoirs of human disease to elucidate the factors that precipitate EID spillover from wildlife to humans (Daszak et al., 2004; Weinhold, 2003).

Hypotheses:

Within the framework of conservation medicine, this research hypothesizes (1) that culturing the 168 bat liver and spleen tissues collected during the 2004 Bangladesh

Nipah virus outbreak will result in the identification of novel viral agents. If the outcome of this research supports hypothesis (1), the discovery of novel viral agents in bat reservoirs of disease may help researchers identify the environmental factors and viral characteristics that separate the viruses that cause disease in humans from the larger pool of bat viruses that do not. Since novel bat-transmitted viruses have often threatened public health, it is important to explore the full diversity of viruses present in bats.

It is further hypothesized that (2) screening a bat sera catalog previously tested for Nipah viral antibody with a newly developed NB virus immunoassay (EIA) will result in the successful detection of NB viral antibody among the bat species in the sera catalog known to carry Nipah viral antibody. If the results of this work support hypothesis (2), temporal or geographic similarities in the seroprevalence patterns of both viruses may be identified in bats. Prevalence pattern similarities may indicate the existence of larger environmental risk factors affecting the prevalence of bat viruses. An increase in the prevalence of a bat viral pathogen may also represent an increased risk that the virus will be zoonotically transmitted to humans. Therefore, similarities in the seroprevalence patterns of Nipah and NB viruses may results in the identification of risk factors for the occurrence of human disease.

Environmental determinants increasing the prevalence of known viruses in bats may be similarly affecting the prevalence levels of viruses yet to be discovered, representing an unquantifiable disease risk to human and veterinary public health (van der Poel et al., 2006). Large scale environmental risk factors of disease may promote the transmission of a range of pathogenic disease agents to humans from many wildlife sources. Since ecologists propose that zoonotic EIDs have strong environmental

determinants and EIDs often occur in unbalanced ecosystems, patterns in the prevalence of bat viruses may also be markers of unhealthy ecosystems. Identifying the environmental factors that may affect human disease risk from bat-transmitted viruses and monitoring changes in bat-viral prevalence may allow public health researchers to identify intervention opportunities to prevent human disease outbreaks and to target EID surveillance strategies to appropriate high-risk environments.

Finally, since bat orthoreoviruses are not known agents of human disease, it is hypothesized that (3) the newly developed EIA will not detect NB viral antibody in the catalog of human specimens previously screened for Nipah viral antibody, although screening for NB viral antibody in humans or bats has not been previously attempted. It is unknown if NB virus is capable of infecting humans. However, the environmental, lifestyle, and behavioral attributes of the Southeast Asian settlements that experienced Nipah virus outbreaks may have also exposed these populations to NB virus since both viruses are maintained in *Pteropus* bat reservoirs in the region (Halpin et al., 2007). If the results of this research support hypothesis (3), it will lend evidence to the supposition that NB virus is not capable of infecting humans despite contact with the bat reservoir of NB virus. It may therefore be concluded that NB virus is not a likely human disease-causing agent. Understanding the range of viral agents capable of causing human disease is important to public heath officials investigating disease outbreaks of unknown etiology.

Chapter II: Literature Review

The Environmental Determinants of Zoonotic Infectious Disease Emergence:

Less developed countries often bear a disproportionate burden of infectious disease morbidity and mortality (Infectious Diseases Society of America., 1992). By citing statistics presented in WHO's *World Health Report: 2004* (WHO Report, 2004), Fauci et al. noted in 2005 in *Emerging Infectious Diseases* that contrary to previous forecasts,

> "…the worldwide impact from infectious diseases remains substantial…[a]lthough annual deaths and lost years of healthy life from infectious diseases have decreased over the past decade, …infectious diseases remain the third leading cause of death in the United States each year and the second leading cause of death worldwide" (Fauci et al., 2005, p. 519).

In 2003, a landmark study by the Institute of Medicine (IOM) attributed infectious disease emergence to several specific risk factors (Smolinski et al., 2003). According to a 2004 article in *Nature* by Morens et al., these risk factors have "social, political, economic, biological, environmental and genetic features" and are defined by the following list:

> "microbial adaptation and change, human susceptibility to infection, climate and weather, changing ecosystems, economic development and land use, human demographics and behavior, technology and industry, international trade and commerce, the breakdown of public health measures, poverty and social inequity, war and famine, lack of political will, and intent to harm" (Morens et al., 2004, p. 245).

Davis and colleagues acknowledge in their Summary and Assessment of the 2001 IOM workshop on the International Aspects of Emerging Infections that "[m]any of these factors lie outside the purview of the health sector, making it difficult to mitigate their

impact on the transmission of infectious diseases and requiring coordinated approaches among various sectors of society for their successful control" (Davis et al., 2001, p. 7).

Morens and colleagues provided an eloquent summary of one of the basic tenets of infectious disease epidemiology, represented by the epidemiologic triangle, in their 2004 article in *Nature*. They point out that "[infectious disease] emergence results from dynamic interactions between rapidly evolving infectious agents and changes in the environment and in host behaviour that provide such agents with favourable new ecological niches" (Morens et al., 2004, p. 242). Daszak et al. explore the determinants of EIDs further in their 2000 article in *Science*. They emphasize that complex relations exist between humans, domestic animals, and wildlife. These relations drive disease emergence and "many wildlife species are reservoirs of pathogens that threaten domestic animal and human health" (Daszak et al., 2000, p. 443). Now considered by many researchers as a basic paradigm of zoonotic EID study, human and wildlife diseases are interdependent, as exemplified by Daszak et al.'s model of the host ecological continuum, depicted in Figure 1 from their 2000 *Science* article (Daszak et al., 2000, p. 443).

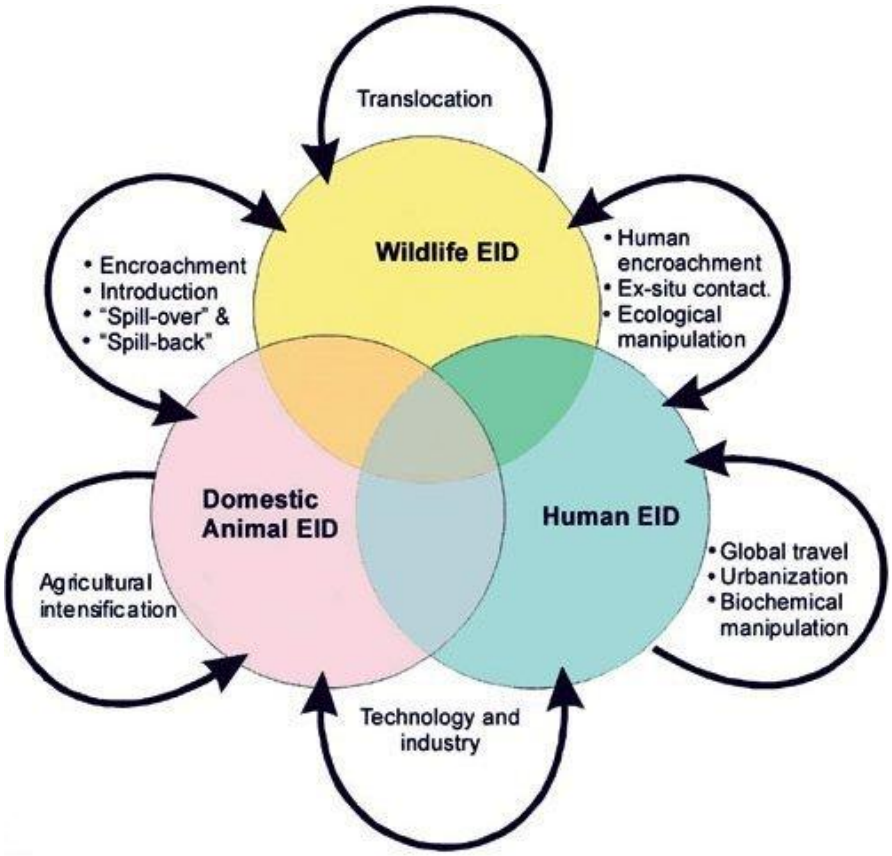

Figure 1. The Host Ecological Continuum. Domestic animal, human, and wildlife EIDs are interdependent and are affected by the factors listed under the arrows. Daszak lists human encroachment, ecological manipulation, and ex situ contact as the factors that enable transmission of infectious agents between wildlife and humans (Daszak et al., 2000, Fig 1, p. 443, *Science*).

Daszak et al., in addition to many other authors, credit Morse for establishing in his 1993 seminal book *Emerging Viruses* (Morse, 1993) that most human EIDs result from the zoonotic transmission of infectious agents from wildlife and that wildlife therefore provide a "vast zoonotic pool" of potential human pathogens (Daszak et al., 2004). The rate of EID transmission to humans may be accelerated by environmental changes, some of which are of human or anthropogenic origin (Daszak et al., 2001; Kruse et al., 2004; Lederberg et al., 1992; McMichael, 2004). A WHO Millennium Ecosystem Assessment Working Group on Land Use Change and Infectious Disease Emergence that

convened in 2002 ranked the top environmental changes of human origin contributing to EIDs (Alcamo et al., 2003; Patz et al., 2004). As cited in a 2004 *Philosophical Transactions of the Royal Society* article by McMichael, the Working Group ranked the following factors in descending order according to the impact they have on EIDs:

> "agricultural development, urbanization, deforestation, population movement, introduced species, biodiversity loss, habitat fragmentation, water and air pollution, road building, impacts of HIV/AIDS, climactic changes, and hydrological changes (including dams)" (McMichael, 2004, p. 1054).

As outlined below, Patz and colleagues described the means by which environmental determinants affect EIDs (Patz et al., 2004). Land use changes often lead to the destruction of wildlife habitats that were once isolated from human populations. This, in turn, results in an exponential growth in human-wildlife interaction and thereby provides exposure to new pathogens for livestock, wildlife, and ultimately, humans (Breed et al., 2005; Chomel et al., 2007; Daszak et al., 2001; Lederberg et al., 1992; Patz et al., 2004).

Deforestation also results in a loss of biodiversity and creates small, genetically homogenous subpopulations of species that are immunologically more susceptible to infectious disease insult (Daszak et al., 2001). Driven by the increasing demands of the human population, global deforestation and habitat encroachment continue at a rate of nearly three percent each year (Patz et al., 2004).

Daszak et al. assert in their 2001 review article in *Acta Tropica* that such land use changes

> "act within a background of pathogen evolution to allow increased transmission between individual hosts, increased contact with new host populations or species, and selection pressure leading to the dominance of pathogen strains adapted to these new environmental conditions" (Daszak et al., 2001, p. 104).

Additionally, volatile weather patterns and increasing temperatures may disrupt the habitats of disease carrying wildlife or predatory species that check the spread of disease vectors, leading to increased concentrations of potential wildlife disease carriers (Patz, 2002). It's often said that "nature abhors a vacuum," and so, infectious diseases emerge in favorable ecological niches created by the dynamic interactions between rapidly evolving infectious agents, changes in the environment, and host behavior (Feldmann et al., 2002; Morens et al., 2004).

Bats-Important Zoonotic Carriers of Emerging Infectious Diseases:

Bats are the third most widely distributed land mammals behind rodents and humans and the approximately 925 species of bats make up around 20% of the 4,600 recognized living mammalian species (Calisher et al., 2006; Halpin et al., 2007; Mackenzie et al., 2003; Torres-Velez & Brown, 2004; S. Wong et al., 2006). Bats serve as reservoirs or hosts of several emerging zoonotic viral diseases in humans. Although rabies (*Rhabdoviridae*) is controlled in dogs and other animals by vaccination in developed countries, in less developed countries bats constitute a major reservoir and transmission source for this virus (Favi et al., 2002). Additionally, bats were identified in 1996 as the reservoir hosts for two new strains of Australian bat lyssavirus, also in the

Rhabdoviridae family, which cause symptoms indistinguishable from rabies in humans (Kuzmin et al., 2006; Warrilow, 2005).

Bats have been implicated as reservoir hosts of the coronaviruses, the virus genus containing the etiologic agent of SARS (Li et al., 2005), and bats are potentially the reservoir host of the filoviruses Ebola and Marburg (Leroy et al., 2005). In addition, bats may play a supportive role in the maintenance or transmission cycles of the following vector borne viruses: the flaviviruses West Nile virus, Kyasanur Forest Disease, and Japanese Encephalitis; the alphaviruses Chikungunya virus and Venezuelan equine encephalitis virus (Mackenzie et al., 2003); and the bunyaviruses, Rift Valley fever virus, and Kaeng Khoi (Osborne et al., 2003). Bats also serve as animal intermediaries of other zoonotic diseases like influenza A virus (Calisher et al., 2006). Investigating the transmission patterns of these viruses using improved diagnostic techniques has lead to the discovery of other bat viruses not yet associated with human disease, including the paramyxovirus Tioman virus (Chua et al., 2001) and the orthoreovirus Pulau virus (Prichard et al., 2006).

Like the lyssaviruses, many bat viruses causing zoonotic EIDs have ancient viral ancestors that coevolved with the predecessors of the bat reservoirs in existence currently (Calisher et al., 2006; Newman et al., 2005). Since several of the cellular receptors and metabolic pathways used by bat viruses were evolutionarily conserved in mammals as species diverged, many viruses maintained in bat reservoirs are biologically capable of interspecies transmission (Breed et al., 2005; Calisher et al., 2006; Newman et al., 2005). Furthermore, bats exhibit migratory patterns and crowded roosting behaviors that provide opportunities for viruses to spread among bat carriers (Calisher et al., 2006).

Experimental infection studies indicate that bats may develop latent viral infections in which viral shedding persists even during seasonal hibernation (Philbey et al., 1998). Combined with their extremely long lifespan (up to 35 years for some bat species), considerable geographic distribution, and diversity in preference of habitat, bats are excellent reservoirs for a variety of viruses (Calisher et al., 2006; Halpin et al., 2007; Mackenzie et al., 2003; Torres-Velez & Brown, 2004; S. Wong et al., 2006).

Bat Taxonomy and Ecology:

Taxonomists classify bats in the kingdom Animalia, phylum Chordata, class Mammalia, and order Chiroptera. The order Chiroptera is further divided into two suborders: Megachiroptera, containing a single family, Pteropodidae (42 genera, 166 species) and Microchiroptera, containing 16 families (135 genera, 759 species). The bats classified in the single family, Pteropodidae, within the Megachiropteran suborder are commonly referred to as flying foxes or Old World fruit bats. The distribution of these bats spans southeast Asia, Australia, the Indian Ocean, and the eastern coast of Africa (Wilson & Reeder, 2005). Seventy-nine percent of species in the family Pteropodidae are Asian and 21% are African (*The Henipavirus Ecology Collaborative Research Group*).

Although much information has been gathered about the role of bats in the suborder Microchiroptera (insectivorous and vampire bats) in the maintenance and spread of viral disease, the Megachiroptera suborder is less well studied (Halpin et al., 2007; Mackenzie et al., 2003). Megachiropterans are the largest bats in the world, eat fruits, flowers, and pollen, and most species navigate at night by eyesight rather than by echolocation (Wilson & Reeder, 2005).

Because many of these species have overlapping habitats with broad geographic distributions, the potential exists for a Megachiropteran bat to transmit a virus throughout a large geographic region with substantial implications for public health (Field et al., 2001; Torres-Velez & Brown, 2004). Figure 2 displays the geographic distribution of the Megachiropteran Old World fruit bats in the family Pteropodidae.

Figure 2. Map of the Distribution of Pteropodidae Bats. Bats in the Pteropodidae family are in the Megachiropteran suborder commonly referred to as Old World fruit bats or flying foxes. Unpublished figure presented with permission from Thomas Ksiazek, Special Pathogens Branch Chief, CDC, Atlanta.

Thus far, researchers have discovered serologic or antigenic evidence of at least 66 viruses in bats to date (Calisher et al., 2006). Under certain environmental circumstances, these viruses cross species barriers, or spillover, to cause zoonotic EIDs in both humans as well as domestic and wild animals. Although many of the viruses are not yet associated with known disease in wildlife or humans, the ability of clinical research to associate a disease with an etiologic agent often lags behind the detection of that agent. We are reminded of this fact by the relatively recent association of a known

metapneumovirus, simply called human metapneumovirus (hMPV), as the causative agent of many lower respiratory tract infections in children (Kahn, 2006). Despite this glaring need for more research into bat viral dynamics, bat ecological studies are often under-funded and underappreciated (Breed et al., 2005; Calisher et al., 2006; Halpin et al., 2007).

Paramyxoviruses in *Pteropus* Bats:

Nipah, Hendra, Menangle and Tioman viruses are newly recognized paramyxoviruses maintained in *Pteropus* bat reservoirs. Nipah, Hendra and Menangle viruses are of great public health importance since they cause human diseases considered EIDs. Other notable viruses in the paramyxovirus family include mumps, measles, and respiratory syncytial virus. Nipah and Hendra viruses are the only two members of the henipavirus genus of the paramyxovirus family. Nipah virus has caused outbreaks of disease in humans via livestock intermediaries in Malaysia (1998) and Singapore (1999) and from direct spillover into human populations from *Pteropus* bats, as is thought to have occurred in the recent outbreaks in Bangladesh (2001, 2003-2007) and in India (2001) (Bellini et al., 2005; Chadha et al., 2006; Hsu et al., 2004). Hendra virus is closely related to Nipah virus. Hendra virus, however, has only caused three cases of human disease after subjects were exposed to the amplified virus through horse intermediaries in Australia in 1995 (Field et al., 2001).

In addition to Nipah and Hendra viruses, *Pteropus* bats are also the suspected reservoir of the paramyxoviruses Menangle and Tioman viruses.

Menangle virus was first identified in 1998 as the causative agent of an outbreak of disease in pigs thought to have caused illness in a cluster of farm workers near Sydney, Australia (Philbey et al., 1998). Conversely, Tioman virus is yet to be associated with human illness. First discovered in Malaysia in 2000 (Chua et al., 2001), Tioman viral antibodies were recently detected in Pteropodinae bats in Madagascar in 2007 (Iehlé et al., 2007), exemplifying the wide geographic distribution of *Pteropus* mediated viruses.

The Epidemiology of Nipah Virus:

Nipah virus infection causes neurological symptoms in humans leading to Nipah viral encephalitic disease. After a 3 to 18-day incubation period, Nipah virus infection usually rapidly progresses from flu-like illness to coma within two days and death within ten days of symptom onset (Ksiazek et al., 1999). Case-fatality ratios vary from 40-92%. About a quarter of the observed Nipah-infected patients have seizures and about 60% become comatose and may require mechanical ventilation, although the full course and spectrum of human disease is unknown (American Public Health Association., 2004). Other than supportive care, there is no treatment for Nipah virus infection; however, Chong and colleagues noted improved outcomes in patients given ribavirin during the Malaysian outbreak (Chong et al., 2001).

Figure 3, accessed June 11, 2007 from the online encyclopedia Wikipedia.org (http://en.wikipedia.org/wiki/Henipavirus#Nipah_virus), maps the locations and the years of the known Hendra and Nipah virus human outbreaks

that occurred in Australia, Bangladesh, India and Malaysia. It also presents the geographic distribution of the *Pteropus* bat reservoirs of Nipah and Hendra viruses. Hendra and Menangle viruses have only caused one outbreak of human disease each, where as Nipah virus outbreaks have often occurred in Southeast Asia. The following sections describe key epidemiological features of the Nipah virus outbreaks detailed in chronological order.

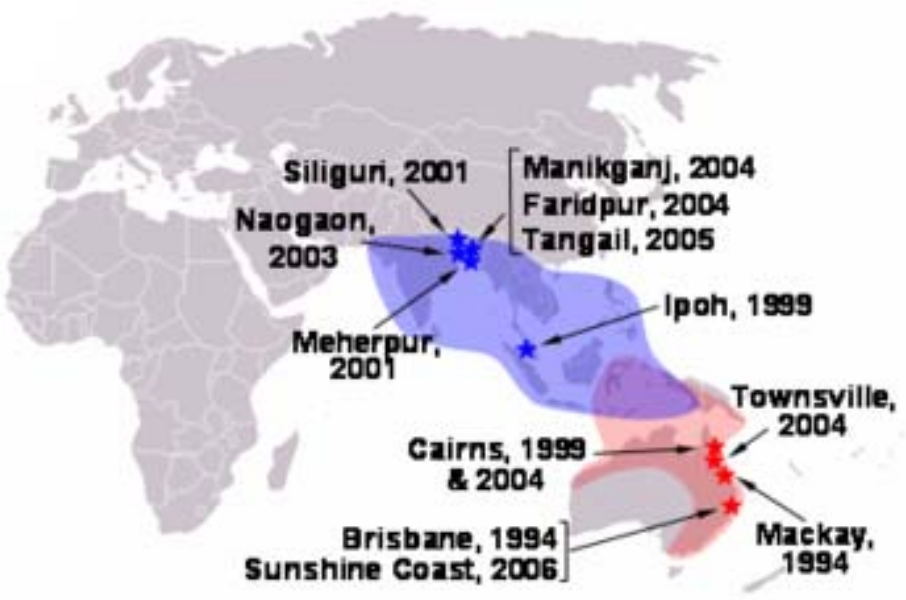

Figure 3. Map of Nipah and Hendra Virus Outbreak Locations. The locations of previous human Nipah virus outbreaks (blue stars) and Hendra virus outbreaks (red stars) and the geographic distribution of the Nipah virus (blue shading) and Hendra virus (red shading) *Pteropus* bat reservoirs (*Henipavirus*, www.en.wikipedia.org/wiki/Henipavirus).

Patterns of Transmission in the First Nipah Virus Outbreak: Malaysia and Singapore, 1998-1999

In 1998, the first diagnosed outbreak of Nipah virus in Malaysia resulted in 104 human deaths out of 265 suspected cases, many of which were attributed to occupational exposure to infected pigs on pig farms or in slaughterhouses (Feldmann et al., 2002). Like humans, infected pigs exhibit primarily neurological symptoms and occasionally respiratory distress known as barking cough, which may have contributed to the spread of Nipah virus among pigs and from pigs to humans via large droplet aerosol formation (K. T. Wong et al., 2002). Since the virus is easily passed from pigs to humans, more than one million pigs were culled during the outbreak, causing great economic distress to the local agricultural industry (Feldmann et al., 2002). Less than a year later, eleven cases and one death from Nipah virus occurred in Singapore among abattoir workers exposed to infected Malaysian pigs (CDC, 1999; Paton et al., 1999).

This outbreak is a prime example of the wildlife, domestic animal, and human host ecological continuum model presented in Figure 1. In theory, the transmission of Nipah virus from wildlife (*Pteropus* flying foxes) to domestic pigs occurred due to an increased overlap between bat habitats and piggeries in Malaysia. At the index farm, fruit orchards were in close proximity to the piggery, allowing pigs close contact with bat urine, feces and partially eaten fruit (Chua, Chua et al., 2002). Subsequent trace back investigation revealed that pig transfers between farms initiated new human outbreaks and genetic sequencing of virus isolates suggested that as little as two instances of spillover from bats to pigs resulted in the successful establishment of infection in pig populations (AbuBakar et al., 2004; Field et al., 2001). Nipah viral antibody was subsequently detected in 21 bats from five Pteropodidae species out of 324 Malaysian

bats captured near locations where Nipah viral infection was reported in pigs (Yob et al., 2001). Furthermore, Nipah virus was successfully isolated from the urine of two Malaysian *Pteropus* bats and from residual bat saliva on a partially eaten piece of fruit (Chua, Koh et al., 2002).

After Malaysia: A Chronology of Nipah Virus Transmission Patterns in Later Outbreaks: India, 2001 and Bangladesh, 2001-2007

Five human Nipah virus outbreaks have been reported in Bangladesh during the winter months in the years 2001-2007. Additionally, eleven isolated cases of Nipah virus encephalitis have appeared in Bangladesh since 2001 ("WHO-Wkly Epi Record," 2004). At the time of this writing, researchers are investigating two confirmed clusters of Nipah virus infection in the Thakurgaon and Gaibandah districts of Bangladesh (S.P. Luby, Personal Communication). Some researchers believe the seasonality of the human outbreaks may be linked to increased viral shedding during *Pteropus* bat pregnancy and parturition or to a seasonal bat habitat or food source ("WHO-Wkly Epi Record," 2004). Figure 4 displays the locations of the human Nipah virus outbreaks that occurred in India and Bangladesh from the years 2001-2004. In Figure 4, Nipah virus outbreaks in 2004 are shown in red while outbreaks in 2001 and 2003 are indicated in blue.

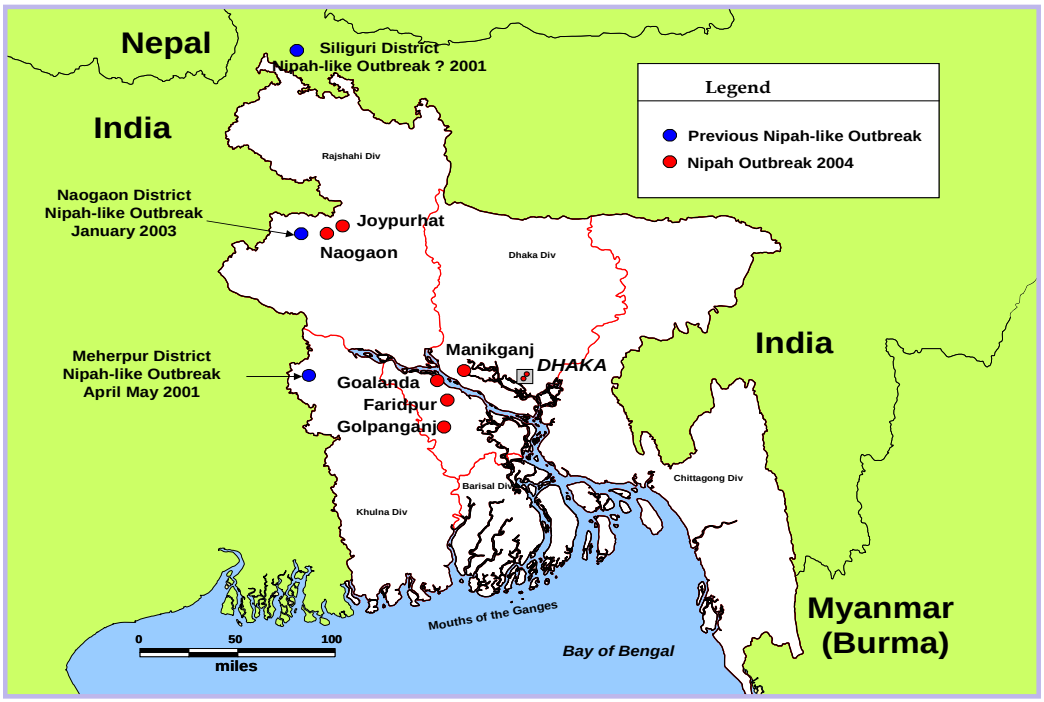

Figure 4. Map of Nipah Virus Outbreak Locations in Bangladesh and India. Outbreaks in red occurred in 2004. Outbreaks in blue occurred in 2001 and 2003. Unpublished figure presented with permission from Joel Montgomery, Special Pathogens Branch EIS Officer.

Nipah Virus Transmission in the 2001 Indian Outbreak:

A 2006 retrospective investigation revealed that Nipah virus caused nine deaths of 18 suspected human cases of encephalitis in February 2001 at healthcare facility in Siliguri, India, near the northern Bangladeshi border. The investigators of the retrospective study attributed the outbreak to nosocomial transmission due to a lack of appropriate barrier nursing practices at the healthcare facility (Chadha et al., 2006). Healthcare-associated transmission also played a role in the Nipah virus outbreak that occurred in Bangladesh in 2004.

Nipah Virus Transmission in the Bangladesh 2001 and 2003 Outbreaks:

In 2003, a retrospective investigation revealed that in April and May of 2001, Nipah virus infection resulted in 9 human deaths of 13 suspected cases in Meherpur, Bangladesh. In January 2003, less than 150 km from Meherpur, 8 fatalities among 12 suspected human cases occurred in Naogaon, Bangladesh. Both outbreaks were concurrently investigated by the same researchers (Hsu et al., 2004). They found that unlike the Malaysian outbreak, as practicing Muslims, most villagers in these areas did not keep pigs as livestock, but wild boars occasionally roamed the villages. Animal serosurveys were conducted as part of the Naogaon investigation. Interestingly, evidence of Nipah virus infection was not detected in pigs, birds, shrews, or rodents; however, two of 44 (4.5%) *Pteropus giganteus* bats collected near the villages were positive for Nipah viral antibody. Contact with bat roosts was epidemiologically linked to Nipah virus infection in the Naogaon outbreak while direct person-to-person spread was determined to be an important mode of transmission in the Meherpur outbreak (Hsu et al., 2004).

Nipah Virus Transmission in the 2004 Bangladeshi Outbreak:

In January 2004, the Manikganj and Rajbari provinces of Bangladesh reported 42 human cases including 14 deaths and, in the following month, the Faridpur province reported 27 fatalities among 36 cases ("WHO-Wkly Epi Record," 2004). Many of those infected in the Rajbari province were males under the age of 15. A case-control study conducted in the area revealed that climbing palm trees to gather fruit, a favorite activity of local boys, was a statistically significant risk factor for Nipah virus infection in this outbreak (Montgomery et al., Publication in Preparation). Local bats in the area roosted

during the day in non-fruit bearing trees, but were observed foraging for fruit at night near patient homes or drinking from clay pots used to collect palm sap from taps placed high in date palm trees to produce a beverage. Since bats shed Nipah virus in urine and saliva, the infected boys likely were exposed to Nipah virus in bat secretions or excretions in the fruit trees or from handling or ingesting fruit partially eaten by bats (Carroll et al., Publication in Preparation).

The Nipah virus outbreak in the Rajbari province was unique. Cases were clustered within households; symptom onset for each case within the household clusters occurred before the minimum known incubation period of three days. This suggests that the household clusters resulted from a single point-source exposure of all those who became symptomatic rather than from person-to-person transmission within the household (Montgomery et al., Publication in Preparation). Furthermore, a greater number of cases than controls reported harvesting or drinking date palm sap, though this was not statistically significant due to the limited size of the outbreak (Carroll et al., Publication in Preparation). As an important part of this outbreak investigation, researchers conducted a Nipah virus seroprevalence study in bats collected near the affected villages. The study revealed that 44% of 109 *Pteropus giganteus* bats were positive for Nipah viral antibody (Carroll et al., Publication in Preparation).

A second Nipah virus outbreak occurred in Bangladesh in 2004 only a month after the outbreak detailed above. A case-control study of the outbreak in the province of Faridpur not only strongly suggested that person-to-person transmission played a predominant role in Nipah viral spread, but also that transmission may have occurred in a bimodal manor, with some singular cases resulting from direct exposure to an unknown

environmental source (ICDDR, 2004). While some cases had no known contact or association with other patients, two cases reportedly resulted from very short patient exposure; specifically, a rickshaw driver became infected after merely transporting a patient to a hospital (ICDDR, 2004). At least six cases in this outbreak manifested a new acute respiratory distress syndrome (similar to the "barking cough" syndrome in pigs) not previously associated with Nipah virus infection in humans which may have contributed to the person-to-person spread of the virus via large droplet formation (ICDDR, 2004).

Nipah Virus Transmission in the 2005 Bangladeshi Outbreak:

One year later, in January 2005, the Tangail District of Bangladesh experienced 11 deaths of 12 Nipah viral encephalitis cases (ICDDR, 2005). A case-control investigation revealed that cases had a statistically significant 7.9 times increased odds of drinking raw date palm sap juice as compared to controls (OR=7.9, 95%CI=1.6-39, P=0.01). Locals prefer to drink this beverage soon after its collection from date palm tree taps before fermentation begins despite the presence of bat excrement often found in the collection vessels (Luby et al., 2006).

A Summary of the Transmission Patterns in the Nipah Virus Outbreaks:

Most striking about the Nipah virus outbreaks described above is that the risk factors, symptom manifestations, and modes of spread varied largely even though these outbreaks occurred in a relatively narrow period in a localized geographic area among human populations with similar characteristics. Modes of Nipah viral spread included amplification due to pig intermediaries combined

with occupational exposure to pigs during slaughter or animal husbandry, food borne transmission via beverages made from palm sap, direct person-to-person transmission, and environmental point-source exposure to the virus via a yet unknown source associated with bats. Table 1 on the following page summarizes the modes of virus spread in each of the bat-transmitted paramyxovirus human outbreaks previously described. One may conclude from the evolving epidemiology of the bat-transmitted paramyxovirus outbreaks highlighted in Table 1 that these viruses are paradigm EIDs. For example, researchers uncover a new aspect of the spectrum of Nipah viral disease with each successive appearance. Much remains to be studied about the etiology and pathogenesis of the bat transmitted paramyxoviruses.

Table 1. Summary of Human Outbreaks caused by Paramyxoviruses Maintained in *Pteropus* Bat Reservoirs.

		Human Cases			
Paramyxovirus	**Outbreak Location**	**Number**	**Case Fatality Ratio**	**Transmission Patterns Identified**	**Bat Sources Examined**
Hendra	Austrailia, 1995	3	100%	Transmitted to humans via horse intermediaries, marked by the sudden die-off of 34 horses	Extensive wildlife serosurveys revealed *Pteropus* bats were the source of the virus
Menangle	Austrailia, 1997	2	0%	Transmitted to humans via pig intermediaries	*Pteropus* bats were bread on the affected farm. Many were seropositive for the viurs
Nipah	Malaysia, Singapore 1998-99	275	38%	Transmitted to humans via pig intermediaries. Over 1 million pigs culled to stop transmission	Wildlife serosurveys revealed *Pteropus* bats were the source of the virus
Nipah	India, 2001	18	50%	Hospital-associated nosocomial transmission	None
Nipah	Bangladesh, 2001	13	69%	Direct, person-to-person spread	None
Nipah	Bangladesh, 2003	12	67%	Contact with bat roosts linked to infection	Seropositive *Pteropus* bats found near village
Nipah	Manikganj and Rajbari, Bangladesh, 2004	42	34%	Cases were young boys, tree climbing a risk factor	Seropositive *Pteropus* bats found near village
Nipah	Faridpur, Bangladesh 2004	36	75%	Bimodal spread: Person-to-person and direct unknown environmental	None
Nipah	Bangladesh, 2005	12	92%	Drinking date palm sap a risk factor	None

The History of Nelson Bay (NB) Virus:

Nelson Bay (NB) virus is similar to the paramyxovirus Tioman virus in the respects that NB virus is also maintained in a *Pteropus* bat reservoir and is also not yet associated with any known disease in wildlife or humans. However, unlike Tioman virus, NB virus is grouped in the Reovirdae family of segmented, double-stranded RNA viruses. Reoviruses infect hosts as diverse as humans, pigs, fish, insects, reptiles, and plants with few restrictions to the range of species in which reoviruses can replicate. There are ten genera in the reovirus family, one of which is the genus orthoreovirus. The genus orthoreovirus is further divided into five species based on host range and the ability of the virus to create large, multinucleated fused cells in culture (syncitia formation), which is an unusual property among viruses lacking the envelope glycoprotein that normally mediates viral cell fusion (Fields et al., 2005). The five species in the orthoreovirus genus include the mammalian orthoreoviruses, avian orthoreoviruses, NB virus, baboon orthoreoviruses, and reptilian orthoreoviruses (Wilcox & Compans, 1982). With the exception of mammalian orthoreoviruses, the four other species of orthoreoviruses generate fusogenic cytopathic effects in cell culture (Fields et al., 2005).

Avian orthoreoviruses inflict great harm on the poultry industry, while mammalian orthoreoviruses are suspected causative agents of meningitis in humans (Zhang et al., 2006). Additionally, researchers are investigating the association between mammalian orthoreovirus infection in infants and cholestatic liver disease (Fields et al., 2005). In human serosurveys, greater than fifty percent of subjects have demonstrated antibody to mammalian orthoreoviruses, with infection often occurring in childhood

(Fields et al., 2005). The NB orthoreovirus species has not been known to cause disease in humans, livestock, or wildlife.

Nelson Bay (NB) virus was first isolated from the heart blood of a *Pteropus poliocephalus* fruit bat in Australia over 30 years ago (G. Gard & Compans, 1970; G. P. Gard & Marshall, 1973). Until 2005, this viral isolate, named simply NB virus, was the sole member of the NB orthoreovirus species and the only known bat orthoreovirus. In 2005, Pulau virus was isolated from a *Pteropus hypomelanus* bat collected from Tioman island in Malaysia (Pritchard et al., 2006). Although it has not been officially characterized yet in the scientific literature, a third bat orthoreovirus, Broome virus, has purportedly been isolated from a *Pteropus alecto* bat in Broome, Australia (Halpin et al., 2007). Pulau virus is closely related to NB virus by phylogenic analysis and Pritchard et al. propose that Pulau virus should be taxonomically classified within the NB orthoreovirus species as Nelson Bay virus-Pulau, or NBV-Pulau (Pritchard et al., 2006).

Nelson Bay Virus Research Opportunities and Their Public Health Significance:

No diagnostic assay currently exists to detect NB virus. The prevalence of this virus in its bat hosts remains unknown (Chua et al., 2001; Dixon, 2007; G. P. Gard & Marshall, 1973; Halpin et al., 2007; Pritchard et al., 2006; S. Wong et al., 2006). In fact, NB virus has been studied so little that fewer than a dozen papers have ever referenced it in the literature since it was first detected in 1970 (G. Gard & Compans, 1970). A serologic assay to detect antibody to NB virus may be a useful diagnostic tool if NB virus were to cross species barriers to cause disease in domestic animals, wildlife, or humans.

Developing an EIA assay to detect NB viral antibody would allow researchers to explore the seroprevalence of NB virus in the *Pteropus* bat reservoir of the virus.

Both NB and Nipah viruses share a common reservoir: *Pteropus* bats in Southeast Asia. If NB virus is effectively transmitted among bat species cohabitating in shared roosts, as has been document for Hendra virus, NB virus may be found throughout the geographic range of *Pteropus* bats. Nipah virus has often been transmitted to humans from *Pteropus* bats in Southeast Asia, but it is not known if NB virus can similarly be passed to humans.

The Southeast Asian human populations that endured the Nipah virus outbreaks detailed previously live in environments that overlap with *Pteropus* bat habitats. Ecologists believe that these habitats are being altered by environmental risk factors such as climate change, deforestation, and shifting agricultural practices. As with other EIDs, these environmental risk factors may similarly encourage the spread of bat-transmitted viral diseases. In addition, the populations that experienced Nipah virus outbreaks have unique behavioral and lifestyle characteristics, such as palm sap consumption and tree climbing, which may put them at risk for contracting other viruses maintained in *Pteropus* bat reservoirs. A human specimen catalog assembled from sera collected during the previous Nipah virus outbreaks in Southeast Asia would contain specimens with antibody evidence of Nipah virus infection. Some of the Nipah virus antibody in the specimen catalog would be attributable to direct human exposure to *Pteropus* bats in Southeast Asia. Screening Nipah virus outbreak-associated human sera for NB viral antibody would therefore investigate the presumption that NB virus has not yet spilled over from *Pteropus* bats to infect humans, unlike the paramyxoviruses Nipah, Hendra,

and Menangle, which have caused terrible disease outbreaks in the region. It is important for public health officials to be aware of the range of viral agents capable causing human disease to effectively investigate outbreaks of unknown etiology.

The prevalence of NB virus in *Pteropus* bats has not been explored. Examining the seroprevalence of NB virus in bat species from Southeast Asia known to carry Nipah viral antibody may elucidate geographic or temporal similarities in the prevalence patterns of both viruses. If seroprevalence pattern similarities are identified, they may indicate that larger environmental risk factors are affecting the prevalence of viruses in bats in the region. Ecologists propose that zoonotic diseases have strong environmental determinants and that they often occur in unbalanced ecosystems (Cook et al., 2004). Therefore, patterns in the prevalence of bat viruses may also be markers of unbalanced ecosystems. Unbalanced ecosystems may promote the zoonotic transmission of a range of pathogenic agents to humans from many wildlife sources (Alcamo et al., 2003). Environmental determinants increasing the prevalence of known viral pathogens in bats may be similarly affecting the prevalence levels of viruses yet to be discovered that represent an unpredictable disease risk to human and veterinary public health (van der Poel et al., 2006). Consequently, identifying the environmental factors that may affect bat viral prevalence may allow public health researchers to target bat-transmitted disease surveillance strategies to appropriate high-risk environments. Careful monitoring of bat viral prevalence rates may allow public health professionals to identify ecologic risk factors for human disease and, ultimately, to develop intervention strategies to prevent or mitigate human outbreaks of bat-transmitted diseases.

Pteropus bats in Southeast Asia are the known reservoirs of several viruses such as Hendra, Tioman, and Menangle, Nelson Bay, Pulau, and Broome viruses. Many of these viruses were only recently discovered in *Pteropus* bats. Other yet unknown viruses may be circulating in these disease hosts. A virus maintained in a *Pteropus* bat reservoir may be easily transmitted to other *Pteropus* bats species throughout the broad geographic distribution of the genus in Southeast Asia, Australia, and coastal Africa. The risk factors leading to seasonal Nipah viral outbreaks in Bangladesh may similarly be exposing Bangladeshi settlements to other *Pteropus* bat viruses. It is therefore important for public health officials to understand the full diversity of viruses present in *Pteropus* bats, especially in Bangladesh, since these bats have often served as sources of devastating Nipah viral disease in the region.

Examining Bangladesh *Pteropus* bat tissue specimens by cell culture for evidence of viral infection may also lead to the identification of novel viral isolates. Bat viruses that cause devastating disease in humans (such as rabies and Nipah viruses) are taxonomically classified in much larger ancient viral families (such as the lyssaviruses and paramyxoviruses). Many of the cellular receptors and metabolic pathways used by viruses in these ancient viral families were evolutionarily conserved as mammalian species diverged. Therefore, many bat viruses are capable of broadly infecting diverse hosts including pigs, horses, and humans. The identification of novel bat viruses, even if they are nonpathogenic to humans, is of public health importance since it may help bat virologist further understand what characteristics separate the bat viruses that cause human disease from the greater pool of bat viruses that do not.

The Importance of Disease Surveillance in Bats within the Framework of Conservation Medicine:

Daszak et al. lamented in 2000 in *Science* that even though wildlife health, human health and ecologic health share common determinants, "[h]istorically, wildlife disease has only been considered important when agriculture or human health has immediately been threatened" (Daszak et al., 2000, p. 443). However, the bat-transmitted viruses discussed in the preceding sections that cause human diseases considered EIDs were once likely confined merely to the bat population before viral spillover led to human infections (Daszak et al., 2000; Steele, 1985). Perhaps some of the morbidity and mortality now associated with bat-transmitted EIDs might have been prevented if researchers at the time had a more acute awareness of the epidemiology and ecology of bat viruses before human disease erupted. Bat serosurveys are quick and cost-effective methods to explore the distribution of viruses maintained in bat reservoirs. However, until a crisis occurs, attention is unfortunately often not focused on such emerging areas of public health research (Fauci, 2001; Torres-Velez & Brown, 2004; Weinhold, 2003). Clearly, public health students interested in EID research must familiarize themselves with these unconventional concepts and exploratory techniques to prepare to tackle the spread of EIDs (Chomel & Osburn, 2006; Daszak et al., 2004; Newman et al., 2005; Torres-Velez & Brown, 2004; Weinhold, 2003).

Focusing on the interaction between the environment, human and non-human hosts, and pathogens, the discipline of conservation medicine strives to define the unpredictable nature of zoonotic EIDs (Daszak et al., 2004). Also called ecological medicine or medical geology, conservation medicine combines the study of human health, animal health, and ecosystem health to describe disease burdens and health

determinants. Professionals in conservation medicine come from diverse disciplines such as microbiology, epidemiology, ecology, wildlife biology, veterinary medicine, and clinical medicine. The discipline is supported by the nonprofit Consortium for Conservation Medicine and includes the Bloomberg School of Public Health of The Johns Hopkins University, the U.S. Geological Survey National Wildlife Health Center, the Harvard Medical School Center for Health and the Global Environment, and the Center for Conservation Medicine at the Tufts School of Veterinary Medicine (Weinhold, 2003). These scientists advocate for more discussion of EIDs within an ecological and social framework within public health schools and institutions (Newman et al., 2005). They anticipate an increased demand for veterinarians, ecologists, epidemiologists, and modelers to work together in interdisciplinary teams since many of the socio-behavioral and environmental risk factors that contribute to EID spread are outside the traditional focus of the public health sector (Daszak et al., 2004; Davis et al., 2001; Steele, 1985).

Researchers using such ecologic approaches to study disease have accurately predicted the occurrence of vector-borne and zoonotic EIDs. Studies like the one recently reported by Peterson and colleagues, which characterized habitat similarities in Ebola virus outbreaks, demonstrate that ecological approaches to EID study benefit our understanding of the epidemiology of EIDs, and are especially useful when little is known about the disease reservoir (Peterson et al., 2004). A timely example, the ongoing Rift Valley fever epidemic in Kenya, Tanzania, and Somalia was predicted weeks before the first human case was recognized by correlating previous outbreaks with climate and precipitation patterns remotely sensed by satellites. Yet despite this forewarning, the

financial and human costs of the outbreak have nevertheless been substantial (Anyamba et al., 2006).

Chapter III: Methodology

Study Description:

The CDC's Special Pathogens Branch in Atlanta investigated many of the Nipah virus outbreaks throughout Southeast Asia detailed in the previous section. The human and bat specimens collected during these investigations were tested by Special Pathogens Branch microbiologists for Nipah viral antibody and preserved in -70ºC archives. In addition to the Nipah virus outbreak specimens, human specimens from Southeast Asia were also submitted to the Special Pathogens Branch to diagnose individual cases of encephalitis of unknown of etiology.

Nelson Bay (NB) virus and Nipah virus share the same reservoir, bats in the genus *Pteropus*. Bat species known to carry Nipah viral antibody may also carry NB viral antibody. Human populations with Nipah viral antibody or with environmental and behavioral risk factors for exposure to *Pteropus* bats may have also been exposed to NB virus. It is important for public health officials to be aware of the range of bat viruses that may infect humans, the distribution of bat-transmitted viruses in humans and in the bat reservoir, and the environmental factors that may affect the prevalence of bat viruses. The seroprevalence of NB virus has not been investigated in wildlife or humans. No diagnostic test to detect the presence of NB virus has previously been developed.

R.W. Compans of Emory University kindly provided a stock of the prototype NB virus strain isolated in 1970 from the Nelson Bay area of New South Wales, Australia (G. Gard & Compans, 1970). This strain was used to develop an enzyme-linked immunoassay (EIA) to recognize immunoglobulin G (IgG) antibody against NB virus in

humans and bats according to an adaptation of previously established protocol (Ksiazek et al., 1999). A new anti-bat IgG conjugate, prepared in a joint effort between the Special Pathogens Branch and Bethyl Laboratories, was used in the NB virus EIA. Previous bat EIAs used a less sensitive and less specific conjugate that recognized the protein A and protein G immunoglobulin components generic to all mammals. In contrast, the anti-bat conjugate used in the NB virus EIA specifically recognized a wide variety of bat species in both the Megachiropteran and Microchiropteran bat suborders (J.B. Oliver et al., Publication in Preparation). A NB virus serum neutralization assay was also developed to confirm the presence of NB virus neutralizing antibody in the EIA positive specimens with sufficient volumes available for testing according to an adaptation of a previously described protocol (Daniels et al., 2001). All laboratory work was performed at the Special Pathogens Branch. These laboratory methods are later described in this chapter.

All human and bat specimens collected during Nipah virus outbreak investigations or previously tested for Nipah virus for exploratory purposes were retrieved from the Special Pathogens Branch archives and organized into bat and human specimen catalogs. Bat serosurveys are quick and cost-effective methods to explore the distribution of viruses maintained in bat reservoirs. To determine if similarities in the seroprevalence patterns of NB virus and Nipah virus exist in the common *Pteropus* bat reservoir of both viruses, the bat sera catalog previously tested for Nipah viral antibody by Special Pathogens Branch microbiologists was screened for NB viral antibody using the newly developed EIA. It is unknown if human NB virus infection has occurred in the populations with environmental, behavioral, and lifestyle attributes that place them at risk for exposure to viruses maintained in *Pteropus* bat reservoirs. It is important for public

health officials to determine if NB virus is capable of infecting humans. Therefore, the human specimen catalog previously tested for Nipah viral antibody by Special Pathogens Branch microbiologists was also screened for NB viral antibody with the EIA. The Nipah and NB viral antibody prevalences in the human and bat catalogs were then compared with respect to the independent variables described in the following section to determine if geographic or temporal seroprevalence pattern similarities exist between the two viruses. If seroprevalence pattern similarities are identified, they may be markers of unbalanced ecosystems and indicate that larger environmental risk factors are affecting the prevalence of bat viruses in Southeast Asia. Identifying the environmental factors that may affect bat viral prevalence may allow public health researchers to target bat-transmitted disease surveillance strategies to appropriate high-risk environments and design intervention strategies to prevent human outbreaks of bat-transmitted disease.

In a joint investigation by the Special Pathogens Branch and the International Centre for Diarrheal Diseases Research (ICDDR) in Dhaka, Bangladesh, spleen and liver tissues from 168 bats of five species were collected from February through May of 2004 during the human Nipah virus outbreak investigation in Bangladesh. The researchers had originally hoped to isolate Nipah virus in cell culture from the bat tissues collected. To examine the ecologic diversity of viruses maintained in bats, the tissues were screened for the presence of viruses by cell culture in the Special Pathogens Branch biosafety level-4 containment lab. Unknown viral isolates detected were identified via electron microscopy performed by C.M. Goldsmith in CDC's Infectious Disease Pathology Branch according to a previously established protocol (Goldsmith et al., 2003). Unknown viral isolates were furthermore characterized by immunoflourescent

microscopic examination. The identification of novel bat viruses, even if they are nonpathogenic to humans, is of public health importance since it may help bat virologists further understand what characteristics separate the bat viruses that cause human disease from the greater pool of bat viruses that do not.

Independent Variables Examined and Tests of Significance:

Nipah and NB virus seroprevalence patterns within the bat and human catalogs were examined with respect to the following characteristics: specimen type, year of specimen collection, and country of specimen collection. Bat seroprevalence was additionally examined with respect to bat species. These characteristics are discussed further for the human and bat catalogs in the following two sections.

For a small cohort of 120 bats collected from Bangladesh in 2004, additional data available on the sex, body size, and GPS location of the bat roost was also studied. The following body measurements, which are often used as surrogate measures of age in bats, were studied: total body length, weight, foot length, ear length, and forearm length (Elangovan et al., 2002). Mean body measurements were calculated within each bat species per sex for the Nipah virus and NB virus seropositive versus seronegative bats and examined for a statistically significant difference by independent T-test using SPSS 15.0 for Windows™ (© SPSS, Inc., 2001, Chicago IL, www.spss.com). Statistically significant differences in the distribution of Nipah or NB virus antibody between male and female bats within each species overall and per bat roost were also examined using Pearson's chi-square analysis or Fisher's exact test, as appropriate. Fisher's exact test is

used instead of Pearson's chi-square when the expected values of any of the cells in a two-by-two contingency table are less than 5 (Hennekens et al., 1987).

A probability value (P value) associated with the tests was calculated to determine if the difference in the distribution was statistically significant. The P value depends on both the magnitude of the difference between the groups and on the sample size. A P value associated with a chi-square test less than or equal to 0.05 means that the observed association between bat gender and the presence of Nipah or NB virus antibody would not be due to chance in 95 out of 100 similar populations. In a two-by-two table, the odds ratio can also be used to compare whether the odds of an outcome occurring is the same within two groups. The magnitude of the odds ratio varies directly with the difference in the distribution of the outcome of interest. For example, an odds ratio of one would imply that the odds of being male versus female were equally as likely in the Nipah or NB virus seropositive versus seronegative bats. An odds ratio greater than one implies that there was a greater odds of one gender being seropositive as compared to the other bat gender. The confidence interval around the odds ratio gives the range that surrounds the odds ratio at a given percent confidence. The width of the range of the confidence interval indicates the amount of variability in the odds ratio due to the effect of sample size (Hennekens et al., 1987). An odds ratio is statistically significant if at a given percent confidence if the confidence interval does not span the null value of one. For a given P value of 0.05, the 95 percent confidence interval by definition does not include the null value (Hennekens et al., 1987).

Characteristics of the Human Specimen Collection:

As displayed in Table 2, the human catalog screened for NB viral antibody consisted of a total of 1,861 specimens previously tested for Nipah viral antibody by Special Pathogens Branch microbiologists. Seventy-three (73) urine specimens were represented in the catalog and all were collected during the 2004 Bangladesh Nipah virus outbreak. The catalog was comprised of 61 cerebral spinal fluid (CSF) specimens. Among them, nine were collected from India (two in 2003 and seven in 2004), 49 from Bangladesh (41 in the year 2004 and four each in the years 2005 and 2006), and three from Indonesia in 2006. The human catalog additionally contained 1,727 sera specimens collected from the years 2001 through 2006 from India, Bangladesh, Thailand, and Indonesia. The human specimens from India in 2001 and from Bangladesh in 2003 and 2004 were collected as part of the Nipah virus outbreak investigations detailed in the preceding chapter. The specimens from Bangladesh and Thailand in 2004 were collected during a retrospective serosurvey of encephalitis among healthcare workers conducted throughout Bangladesh and Thailand. The remaining specimens were submitted to the Special Pathogens Branch for Nipah virus testing to investigate clusters of encephalitis due to unknown agents.

Table 2. Characteristics of the Human Specimen Catalog

Urine		CSF		Serum	
Specimen Group	**Number**	**Specimen Group**	**Number**	**Specimen Group**	**Number**
Bangladesh, 2004	73	India, 2003	2	India, 2001	26
Total	**73**	India, 2004	7	India, 2003	13
		Bangladesh, 2004	41	India, 2004	7
		Bangladesh, 2005	4	Bangladesh, 2003	234
		Bangladesh, 2006	4	Bangladesh, 2004	1,133
		Indonesia, 2006	3	Bangladesh, 2005	34
		Total	**61**	Bangladesh, 2006	23
				Bangladesh/Thailand, 2004	160
				Thailand, 2004	77
				Indonesia, 2006	20
				Total	**1,727**

All human patient identifiers were previously stripped from the collection. The CDC human subjects ethics committee Institutional Review Board (IRB) did not require further review of the protocol since all specimens were anonymized and did not consider this to be human subjects research under 45CFR46.102(f) of the Code of Federal Regulations. The Georgia State University Institutional Review Board determined the protocol to be exempt research according to 45CFR46.101(b) (GSU IRB Protocol H07178). Protocol approval was granted by the Georgia State University Internal Biosafety Committee (Protocol Approval B07004).

Characteristics of the Bat Sera Collection:

The catalog of bat specimens screened for antibody evidence of NB virus consisted of 2,323 sera specimens. All bat specimens had previously been tested for Nipah viral antibody by Special Pathogens Branch microbiologists. The bat sera specimens were collected in the years 1993 through 2006 from Bangladesh, Thailand, Cambodia and Singapore or from wild-caught bats from locations throughout Southeast

Asia acquired by bat conservation groups. Detailed specimen histories were lacking for the specimens submitted from the bat conservation groups, and so it was unknown which of the specimens from these groups were collected from wild-caught bats, which specimens were collected from bats raised in captivity, or if specimens from the same bat were tested multiple times.

Many of the bat specimens screened were originally collected as ecological serosurveys for Nipah virus in bats. The bat specimens from Bangladesh in 2003 and 2004 were collected as part of the Nipah virus human outbreak investigations detailed in the previous chapter. Twenty-seven species, many of which are rare or endangered, were represented in the catalog. Table 3 shows the distribution of the bat specimens examined by specimen group.

Table 3. Characteristics of the Bat Sera Catalog

Specimen Group	**Number**
Bat Conservancy Groups, 1993-2006	1,569
Cambodia, 2000	244
Cambodia, 2001	109
Singapore, 2000	3
Thailand, 2002	175
Bangladesh, 2003	56
Bangladesh, 2004	168
Total	**2,323**

Table 4 on the following page displays the distribution of the bat specimens screened by species, location(s) of specimen collection, and gives the species taxonomic classification.

Table 4. Distribution by Species of the Bat Sera Catalog. Taxonomy and sera specimen collection location distributed by species of the 2,323 bat specimens screened. Species marked with an asterisks were previoulsy reported to carry Nipah viral antibody.

Genus and Species	Suborder	Family	Subfamily	Sera Collection Location(s)	Number Screened
Unknown	Unknown	Unknown	Unknown	Singapore and Bat Conservancy Groups	6
Artibeus jamaicensis	Microchiroptera	Phyllostomidae	Stenodermatinae	Bat Conservancy Groups	3
Chaerephon plicata	Microchiroptera	Mollosidae	n/a	Cambodia	110
*Cynopterus brachyotis**	Megachiroptera	Pteropodidae	Pteropodinae	Cambodia and Bat Conservancy	56
Cynopterus sphinx	Megachiroptera	Pteropodidae	Pteropodinae	Bangladesh and Cambodia	16
Cynopterus species unknown	Megachiroptera	Pteropodidae	Pteropodinae	Bangladesh	3
Epomophorus wahlbergi	Megachiroptera	Pteropodidae	Pteropodinae	Bat Conservancy Groups	10
Eidolon helvum	Megachiroptera	Pteropodidae	Pteropodinae	Bat Conservancy Groups	35
Emballonura monticola	Microchiroptera	Emballonuridae	n/a	Thailand	12
*Eonycteris spelaea**	Megachiroptera	Pteropodidae	Macroglossinae	Bangladesh and Thailand	55
*Hipposideros larvatus**	Microchiroptera	Rhinolophidae	Hipposiderinae	Thailand	63
Hipposideros species	Microchiroptera	Rhinolophidae	Hipposiderinae	Bangladesh	1
Megaderma Lyra	Microchiroptera	Megadermatida	n/a	Bangladesh	10
Megaderma species unknown	Microchiroptera	Megadermatida	n/a	Bangladesh	1
Myotis hasseltii	Microchiroptera	Vespertilionidae	Vespertilioninae	Cambodia	1
Myotis mystacinus	Microchiroptera	Vespertilionidae	Vespertilioninae	Cambodia	5
Myotis species unknown	Microchiroptera	Vespertilionidae	Vespertilioninae	Cambodia	1
Phyllostomus species	Microchiroptera	Phyllostomidae	Phyllostominae	Bat Conservancy Groups	25
Pipistrellus aegyptius	Microchiroptera	Vespertilionidae	Vespertilioninae	Bat Conservancy Groups	19
Pteropus conspicillatus	Megachiroptera	Pteropodidae	Pteropodinae	Bat Conservancy Groups	5
*Pteropus giganteus**	Megachiroptera	Pteropodidae	Pteropodinae	Bangladesh and Bat Conservancy	274
*Pteropus hypomelanus**	Megachiroptera	Pteropodidae	Pteropodinae	Thailand and Bat Conservancy Groups	317
*Pteropus lylei**	Megachiroptera	Pteropodidae	Pteropodinae	Cambodia and Thailand	132
*Pteropus poliocephalus**	Megachiroptera	Pteropodidae	Pteropodinae	Bat Conservancy Groups	56
*Pteropus pumilus**	Megachiroptera	Pteropodidae	Pteropodinae	Bat Conservancy Groups	170
*Pteropus rodricensis**	Megachiroptera	Pteropodidae	Pteropodinae	Bat Conservancy Groups	269
*Pteropus vampyrus**	Megachiroptera	Pteropodidae	Pteropodinae	Bat Conservancy Groups	442
Pteropus species unknown	Megachiroptera	Pteropodidae	Pteropodinae	Cambodia and Bat Conservancy	11
*Rousettus leschenaulti**	Megachiroptera	Pteropodidae	Pteropodinae	Bangladesh	30
Rousettus species unknown	Megachiroptera	Pteropodidae	Pteropodinae	Bangladesh and Bat Conservancy	63
Scotophilus heathi	Microchiroptera	Vespertilionidae	Vespertilioninae	Thailand	3
*Scotophilus kuhli**	Microchiroptera	Vespertilionidae	Vespertilioninae	Cambodia	43
Tadarida species unknown	Microchiroptera	Mollosidae	Murinae	Cambodia	9
Taphozous melanopogon	Microchiroptera	Emballonuridae	n/a	Cambodia	56
Taphozous theobaldi	Microchiroptera	Emballonuridae	n/a	Camboida	11
*Species previously reported to carry Nipah viral antibody				**TOTAL**	**2,323**

Laboratory Methods:

Nelson Bay (NB) Virus and Unknown Virus Isolation Techniques:

Ten ml of a 10^{-2} dilution in Hanks Balanced Salt Solution (HBSS) of NB virus (prototype strain isolated from the Nelson Bay area of New South Wales, Australia provided by R.W. Compans of Emory University) was inoculated into 850 cm^2 roller bottles of confluent Vero E6 cells and incubated at 37ºC for 1 hour. Rollers were then re-fed with 150 ml of Eagle's Minimum Essential Medium (EMEM) with 2% fetal calf serum and incubated at 37ºC. Rollers were examined daily for development of cytopathic effects (CPE). Successful infection was established by indirect immunofluorescence assay (IFA) examination of the slides using mouse anti-NB virus hyperimmune ascitic fluid (HMAF) as a primary antibody (1:100 dilution). Slides were incubated with 25 µl of the HMAF solution for 30 min at 37ºC in a humidified incubator, washed with 0.01M PBS at pH 7.2, and allowed to dry. 25 µl of a florescent-labeled anti-mouse antibody commercially available from Cappel (rehydrated per manufacturer's instructions and used at a 1:40 dilution) was then applied to the slides. Slides were again incubated for 30 min at 37ºC, washed with 0.01M PBS at pH7.2, counterstained with eriochrome black-T, and allowed to dry. Slides were examined using florescent microscopy.

To screen for virus in the spleen and liver tissues from the 168 bats collected in 2004 from Bangladesh, the tissues were ground using a mortar and pestle in HBSS and alundum in the biosafety level-4 containment lab. The resulting suspension was centrifuged and 100 µl of this supernatant was used to inoculate confluent Vero E6 cells

in 25 cm^2 flasks and incubated at 37ºC for 1 hr. After incubation, flasks were re-fed with EMEM with 2% fetal calf serum and incubated at 37ºC. When CPE developed, cells were scraped from the rollers, fixed to slides, inactivated with 2×10^6 rads of gamma irradiation (Co-60 source), and examined by IFA.

Electron Microscopy of Unknown Virus Isolates:

Electron microscopy identification of unknown viral isolates detected in the tissues from the 168 bats collected in 2004 from Bangladesh was performed by C.M. Goldsmith of the Infectious Disease Pathology Branch, CDC according to a previously established protocol (Goldsmith et al., 2003). Essentially, supernatant from infected Vero E6 flasks displaying CPE were fixed in 2.5% gluteraldehyde (1:1) for 5 hrs. The fixative was decanted and replaced with sodium phosphate buffer, pH 7.2 and inactivated by gamma irradiation (2×10^6 rads using a Co-60 source). The resulting specimens were embedded in epoxy and thin sections were stained with uranyl acetate and lead citrate for visualization.

Nelson Bay (NB) Virus Mouse Hyperimmune Ascitic Fluid (HMAF) Development:

Mouse antibodies to NB virus were produced by P.E. Rollin of the Special Pathogens Branch according to a previously detailed protocol (Brandt et al., 1967). Ten pathogen-free female mice were immunized by two intraperitoneal inoculations of a 0.3% Beta-propiolactone inactivated 10% suckling mice brain suspension of prototype NB virus in Freund's complete adjuvant two weeks apart. On day 28, the mice were injected with a Sarcoma TG-180 cell suspension by the intraperitoneal route. Mice were then

tapped on day 30. The resulting NB virus HMAF was pooled, inactivated by gamma irradiation (2×10^6 rads using a Co-60 source), and tested for reactivity by checkerboard cross-titration EIA. All animal procedures were performed in accordance with CDC's Interagency Animal Care and Use Committee approved protocols in the biosafety level-4 laboratory.

Indirect Nelson Bay (NB) Virus IgG EIA Development:

R.W. Compans of Emory University kindly provided a stock of the prototype NB virus strain isolated in 1970 from the Nelson Bay area of New South Wales, Australia (G. Gard & Compans, 1970). This strain was used to develop an enzyme-linked immunoassay (EIA) to recognize immunoglobulin G (IgG) antibody against NB virus in humans and bats according to an adaptation of previously established protocol (Ksiazek et al., 1999). Briefly, NB virus antigen (prototype strain) was extracted from infected Vero E6 cells using detergent, inactivated by gamma irradiation (2×10^6 rads using a Co-60 source), and sonnicated. Antigen from uninfected Vero E6 cells was similarly prepared and used as a negative control against nonspecific sera binding. 100 µl per well of the antigens was absorbed to 96-well plates (BD Falcon Cat No. 353910) at a dilution of 1:1000 in 0.01M PBS, pH 7.2 as previously determined by checkerboard cross-titration with NB virus positive hyperimmune mouse ascitic fluid (HMAF) and allowed to incubate overnight at 4ºC. The plates were then washed three times with 200 µl of a 0.01M PBS and 0.1% Tween-20 wash buffer solution at pH 7.2 and 100 µl per well of the unknown human or bat sera was applied to the plates in 4-fold serial dilutions starting with an initial dilution of 1:100. All sera was also inactivated by gamma cell irradiation

(2×10^6 rads with a Co-60 source) and diluted in serum diluent (0.01M PBS pH 7.4, 0.5% skim milk, and 0.1% Tween-20). The sera was allowed to bind to the antigen for 1 hour at 37ºC in a humidified incubator, plates were then washed three times with 200 µl of wash buffer solution, and 100 µl per well of the appropriate commercially available conjugate was added. Anti-bat IgG conjugate (H+L) from Bethyl Laboratories (rehydrated in 1 ml of 50:50 solution of glycerol:water) was applied at a 1:2000 dilution in serum diluent for the bat sera screening or anti-human IgG (H+L) from Accurate at a 1:4000 dilution in serum diluent was applied for the human sera screening. Plates were again incubated for 1 hr at 37ºC, washed, and 100 µl per well of ABTS substrate solution commercially available from Kirkgard and Perry Laboratories and prepared per manufacturer's instructions was applied. After a final 30 min incubation at 37ºC, plates were read at 405 nm and 495 nm absorbance. A positive reaction was defined as a titer greater than or equal to 400 and a sum optical density (OD) greater than or equal to a three-fold standard deviation increase from the mean sum OD of the negative sera after subtracting nonspecific sera binding to the negative control antigen.

Nelson Bay (NB) Virus Serum Neutralization Assays:

Considered the gold standard for the detection of neutralizing antibodies to a virus in question, serum neutralization assays were performed on the NB virus EIA positive specimens with sufficient volumes available. Serum samples were heated for 30 min at 56°C and then were titrated with five dilutions (1:10; 1:40, 1:160, 1:640, and 1:2560) in a 24-well culture plate. An equal volume of NB virus (at 100 times the 50% tissue culture infective dose ($TCID_{50}$) in 100 µl) was added to all sera, and the plate was incubated for 1 hr at 37°C. Vero E6 cells (5×10^5 cells/ml in EMEM with 5% FBS) were added to all

wells, and the plates were incubated at 37°C for 7 days in a CO_2 chamber. All wells were examined daily for characteristic cytopathic effects (CPE) showing large syncytia under a microscope in each well. The number of virus-positive wells was confirmed after fixation with two successive changes of 5% glacial acetic acid in absolute ethanol for 30 min each. Plates were gamma irradiated at 2×10^6 rads using a Co-60 source and then stained with 1 ml of crystal violet stain for 15 min, washed with deionized water, and plaques were counted. Toxicity of the sera for Vero cells was observed on uninfected cells in the presence of 1:10 serum dilution. Normal mouse and NB virus HMAF were used as positive and negative serum controls, respectively. The neutralization titer of each sample was defined as the last serum dilution in which at least half of the monolayer was intact ($TCID_{50}$).

Chapter IV: Results

Nipah Virus Seroprevalence Patterns in the Human Specimen Collection:

As was determined by the previous Nipah virus serologic testing conducted by the Special Pathogens Branch, none of the 73 human urine specimens collected during the 2004 Bangladesh Nipah virus outbreak were Nipah virus seropositive. Of the 61 CSF specimens in the human specimen catalog, only one specimen collected during the 2004 Bangladesh Nipah virus outbreak investigation was Nipah virus antibody positive.

Out of the 1,727 human serum specimens in the collection screened for NB viral antibody, only 46 were Nipah virus antibody positive (2.7%). Thirty-eight (38) of the 46 Nipah virus antibody positive human serum specimens were obtained from the Nipah virus outbreak investigations conducted in Bangladesh in the years 2003, 2004, and 2005. This data is displayed in Table 5. Aside from the Bangladesh outbreak specimens, the only other Nipah virus antibody positive human sera in the catalog was collected from the 2001 Nipah virus outbreak in India and from the retrospective serosurvey of encephalitis among healthcare workers conducted throughout Bangladesh and Thailand in 2004. Seven of the 26 sera (26.9%) collected during the 2001 Indian Nipah virus outbreak were antibody positive, as was one specimen of the 160 (0.6%) from the 2004 healthcare worker serosurvey in Bangladesh and Thailand.

Table 5. Distribution of the Human Sera Screened by Group.

Group	Number of Sera in Group	Percent of the Total Sera in Group	Number of Seropositive in Group	Percent of Group Nipah Positive	Percent of the Total Seropositive in Group
India, 2001	26	1.5%	7	26.9%	15.2%
India, 2003	13	0.8%	-	-	-
India, 2004	7	0.4%	-	-	-
Bangladesh, 2003	234	13.5%	9	3.8%	19.6%
Bangladesh, 2004	1133	65.6%	25	2.2%	54.3%
Bangladesh, 2005	34	2.0%	4	11.8%	8.7%
Bangladesh, 2006	23	1.3%	-	-	-
Thailand, 2004	77	4.5%	-	-	-
Bangladesh/ Thailand, 2004	160	9.3%	1	0.6%	2.2%
Indonesia, 2006	20	1.2%	-	-	-
Total Percent	**1,727**	**100%**	**46**	**2.7%**	**100%**

Nipah Virus Seroprevalence Patterns in the Bat Sera Collection:

Overall, 200 (8.6%) of the bat serum specimens were Nipah virus antibody positive, as determined by previous laboratory testing by the Special Pathogens Branch. Table 6 gives the distribution of the bat specimens screened by specimen group. As is highlighted in yellow in Table 8, note that even though the 168 specimens in the 2004 Bangladesh bat cohort made up only 7.2% of the entire bat catalog of 2,323 specimens, 24.5% of the 200 Nipah virus antibody positive bat specimens belonged to the cohort. The 2004 Bangladesh bat specimens were collected as part of the ecological serosurveys for Nipah virus in bats conducted during the human Nipah virus outbreak investigation in Bangladesh in 2004 (Carroll et al., Publication in Preparation). Of note, the virus

isolation screening results reported by this research was conducted on the spleen and liver tissues from the 168 bats in 2004 Bangladesh cohort.

Table 6. Distribution of the Bat Sera Screened by Specimen Group.

Group	Number of Sera in Group	Percent of the Total Sera in Group	Number of Nipah Virus Seropositive in Group	Percent of Group Nipah Positive	Percent of the Total Nipah Virus Seropositive in Group
Bat Conservancy Groups, 1993-2006	1,569	67.5%	143	9.1%	71.5%
Cambodia, 2000	244	10.5%	5	2.0%	2.5%
Cambodia, 2001	109	4.7%	-	-	-
Singapore, 2000	3	0.1%	-	-	-
Thailand, 2002	175	7.5%	1	0.6%	0.5%
Bangladesh, 2003	56	2.4%	2	3.6%	1.0%
Bangladesh, 2004	168	7.2%	49	29.2%	24.5%
Total Percent	**2,323**	**100%**	**200**	**8.6%**	**100%**

As displayed in Table 8, the bulk of the 2,323 specimens screened (67.5%) were collected from bat conservation groups and 143 (9.1%) of these were Nipah virus antibody positive. Since detailed specimen histories from the bat conservation groups were lacking, it was unknown if these Nipah virus antibody positive bats were captured in the wild, if they were raised in captivity, or if multiple specimens from the same animal were screened. Of the 353 sera from Cambodia (collected in 2000 and 2001), 1.4% were positive for Nipah viral antibody. Of note, the Cambodian bat specimens screened were obtained from animals later prepared and served as a local delicacy in restaurants in Phnom Penh (Olson et al., 2002). None of the three specimens from Singapore were positive and only one of the 175 specimens (0.6%) collected from Thailand was Nipah virus antibody positive.

The two remaining Nipah virus antibody positive specimens were collected during the bat serosurveys conducted by the investigators of the 2003 Bangladesh Nipah virus outbreak. Of the 56 bat specimens in the panel from Meherpur and Naogaon, Bangladesh collected in 2003 by Hsu et al., two *Pteropus giganteus* species bats from Naogaon (3.6%) were Nipah virus seropositive (Hsu et al., 2004). In comparison, the 2004 Bangladesh Nipah virus outbreak investigation in the town of Goalando in the Rajbari district revealed that 48 of the 109 (44.0%) *P. giganteus* bats collected were Nipah virus antibody positive, as was one of the 30 (3.3%) *Rousettus leschenaulti* bats collected (Carroll et al., Publication in Preparation). Including all five bat species collected during the 2004 Bangladesh outbreak, 29.2% of the 168 total bats were Nipah virus antibody positive (Carroll et al., Publication in Preparation).

All 200 Nipah virus antibody positive bat specimens were collected from Old World fruit bats in the Pteropodinae subfamily. Of the 1,889 Pteropodinae bats screened, 10.6% were Nipah virus antibody positive. Moreover, 199 out of the 200 Nipah virus antibody positive bats belonged to the *Pteropus* genus while the remaining Nipah virus antibody positive specimen was from a *Rousettus leschenaulti* bat. A pie chart distribution by species of the 200 Nipah virus antibody positive bats is displayed in Figure 5. Nipah viral antibody was present in the following seven bat species in the bat sera catalog: *P. giganteus*, *P. vampyrus*, *P. rodricensis*, *P. pumilus*, *P. lylei*, *P. hypomelanus*, and *R. lechenaulti*. Sixty-seven percent (67%) of the Nipah virus antibody positive bats were from the *P. giganteus* and *P. vampyrus* species, though these two species made up only 30.8% of the entire collection.

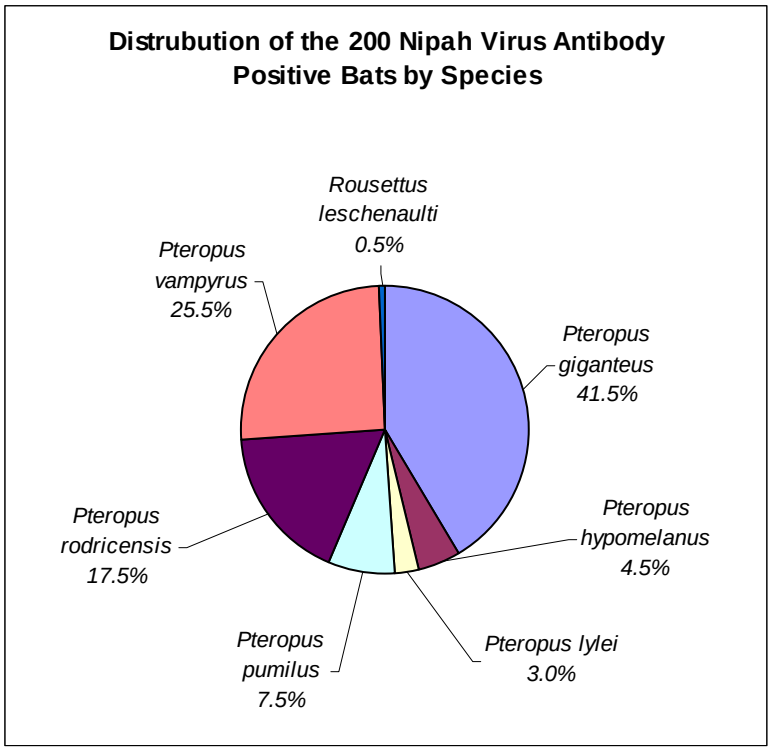

Figure 5. Distribution of the Nipah Virus Antibody Positive Bat Specimens by Species.

Nelson Bay (NB) Virus EIA Serology Results:

None of the 1,861 human specimens screened were positive for NB virus. Fourteen (14) NB virus antibody positive bats were detected out of 2,323 specimens screened (0.6%). As discussed in the Laboratory Methods section, the NB virus sum OD cut off values for the bat and human assays were calculated based on a three-fold standard deviation increase from the mean sum OD of the negative sera after subtracting nonspecific sera binding to the negative control antigen. The calculated NB virus sum OD cutoff values were 0.85 for the bat sera screening and 1.30 for the human sera screening. The bat and human sera sum OD values were normally distributed. The sum

OD frequency distributions are displayed as histograms in Figures 6 and 7 for the human and bat screening, respectively.

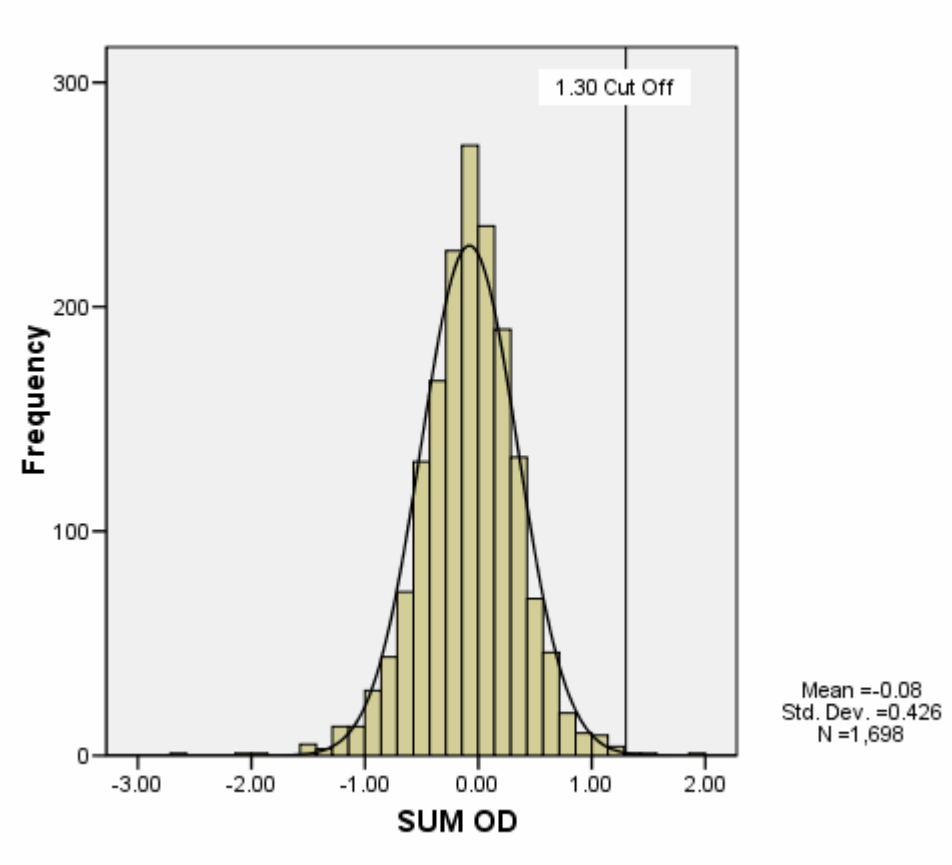

Figure 6. Human NB Virus EIA Sum OD Frequency Distribution Histogram. The frequency distribution of the sum OD values generated by the NB virus EIA of the human specimens.

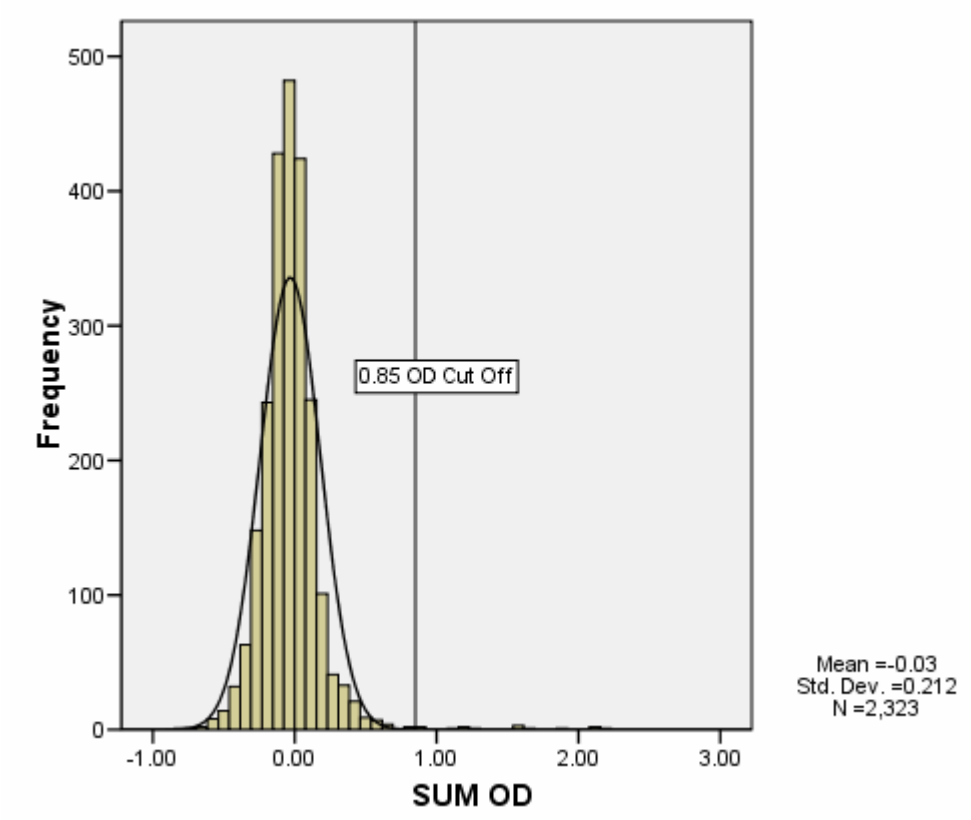

Figure 7. Bat NB Virus EIA Sum OD Frequency Distribution Histogram. The frequency distribution of the sum OD values generated by the NB virus EIA of the bat specimens.

Nelson Bay (NB) Virus Serum Neutralization Assay Results:

A sufficient quantity of sera from nine of the 14 NB virus seropositive bat specimens was available for serum neutralization testing. All specimens tested by serum neutralization were positive for the presence of neutralizing antibodies to NB virus. Seven of the nine specimens had neutralizing antibody titers at a dilution of 1:2,560. One of the remaining two sera specimens tittered to a dilution of 1:640 and the other specimen had neutralizing antibody only to a dilution of 1:160. The antibody titer dilution of the serum specimen corresponds to the concentration of neutralizing antibody present in the

sample: the greater the antibody titer dilution, the more antibody capable of neutralizing the NB virus present in the sample.

Nelson Bay (NB) Virus Seroprevalence Patterns in the Specimen Collections:

Since no human specimens were NB virus antibody positive, no seroprevalence patterns were detected in the human specimen catalog. Therefore, the human catalog could not be further examined for pattern similarities in the seroprevalences of Nipah and NB viruses. Of the 14 NB virus antibody positive bat specimens detected, ten were collected from *R. leschenaulti* species bats, one was collected from a *Rousettus* bat of unknown species, two were collected from *P. giganteus* bats, and the final positive specimen was collected from a *P. vampyrus* species bat. The 13 NB virus seropositive *Rousettus* and *P. giganteus* bats detected in the screening belonged to the cohort of 168 bat specimens collected during the 2004 Bangladesh human Nipah virus outbreak investigation; the one remaining NB virus seropositive *P. vampyrus* specimen was collected in 2000 from an unknown location by a bat conservation group. Although it cannot be confirmed, it is likely that the bat conservation group obtained this specimen from a bat captured in Malaysia.

Nipah and Nelson Bay (NB) Virus Seroprevalence Pattern Similarities in the Bat Sera Collection:

As previously displayed in the pie chart in Figure 5, seven of the 27 bat species represented in the collection were Nipah virus seropositive. These seven species were previously identified in the literature as Nipah viral antibody carriers and all were taxonomically classified in the Pteropodinae subfamily within the Megachiropteran

suborder. Nelson Bay (NB) viral antibody was successfully detected in three of the seven Nipah seropositive bat species (*P. giganteus*, *P. vampyrus*, and *R. lechenaulti*). Table 7 on the following page presents the distribution of the Nipah and NB virus serology positive specimens by species.

Table 7. Distribution of the NB virus or Nipah Virus Seropositive Specimens by Species. All species listed were previously reported Nipah virus seropositive in the literature.

Genus and Species	Sera Collection Location(s)	Number Screened	Percent of Species in Total Collection	Number Nipah Virus Anitbody Positive	Percent of Species Nipah Virus Antibody Positive	Number NB Virus Antibody Positive	Percent of Species NB Virus Antibody Positive
Pteropus giganteus	Bangladesh and Bat Conservancy	274	11.8%	83	30.3%	2	0.7%
Pteropus hypomelanus	Thailand and Bat Conservancy Groups	317	13.7%	9	2.8%	-	-
Pteropus lylei	Cambodia and Thailand	132	5.7%	6	4.5%	-	-
Pteropus pumilus	Bat Conservancy Groups	170	7.3%	15	21.4%	-	-
Pteropus rodricensis	Bat Conservancy Groups	269	11.6%	35	13.0%	-	-
Pteropus vampyrus	Bat Conservancy Groups	442	19.0%	51	11.5%	1	0.2%
Rousettus leschenaulti	Bangladesh	30	1.3%	1	3.3%	10	33.3%
Rousettus species unknown	Bangladesh and Bat Conservancy	63	2.7%	-	-	1	1.6%
	Total	**1,697**		**200**		**14**	

As Table 7 displayed, 10 of the 30 (33.3%) total *R. leschenaulti* specimens screened were NB virus antibody positive. In contrast, the Nipah virus seroprevalence in the 30 *R. leschenaulti* bats was 3.3%. In the other two bat species found to carry NB viral antibody, the NB viral seroprevalence was merely 0.7% of the 274 *P. giganteus* screened and 0.2% of the 442 *P. vampyrus* screened. In comparison, the Nipah viral antibody prevalence in the *P. giganteus* bats screened was 30.3% and 11.5% in the *P. vampyrus* bats.

Out of the 14 total NB virus seropositive bats detected in the screening, the 13 NB virus seropositive *Rousettus* and *P. giganteus* species bats belonged to the cohort of 168 bat specimens collected during the 2004 Bangladesh human Nipah virus outbreak investigation. The only *Rousettus* specimens in the catalog screened were collected during the 2004 Bangladesh Nipah virus human outbreak investigation from a single bat roost near the location of the human Nipah virus cases. Bat roost GPS locations, gender, and bat body measurements were recorded in the field for 120 of the 168 bats in the Bangladesh 2004 cohort. Further statistical characterization of the Nipah and NB virus antibody positive *R. leschenaulti* and *P. giganteus* bats from the Bangladesh 2004 cohort was possible using this data.

Of the 109 *P. giganteus* bats collected in Bangladesh in 2004, only two (1.8%) were NB virus antibody positive (one male and one female). Both of these bats, however, also tested positive for Nipah viral antibody. A *R. leschenaulti* female and a *Rousettus* bat of unknown species and unknown sex additionally tested positive for both Nipah and NB viral antibody in the Bangladesh 2004 cohort. Therefore, four of the 14

(28.6%) total NB virus seropositive bats identified in this screening were also simultaneously positive for Nipah viral antibody.

Nipah and Nelson Bay (NB) Virus Seroprevalence Pattern Similarities with Respect to Bat Gender:

As displayed in Table 8, seven of the 14 (50.0%) *R. leschenaulti* females collected in the 2004 Bangladesh human Nipah virus outbreak investigation were positive for NB viral antibody, as were three of the 16 (18.8%) males. Therefore, a greater percentage of *R. leschenaulti* females as compared to males were NB virus antibody positive, but due to the small sample size, this difference in the distribution of seroprevalence by sex was not statistically significant (P=0.122) by Fisher's exact test. In terms of the odds ratio, there was a 4.3 times greater odds of being female than male among the *R. leschenaulti* seropositive bats, but the odds of being female were not statistically significant (95% confidence interval=0.8-22.2).

Table 8. NB Virus Seroprevalence by Gender in the *Rousettus leschenaulti* Bats. The bats were collected near the 2004 human Nipah virus outbreak in Bangladesh from a single bat roost. Differences in the distribution by gender were not statistically significant.

Species: *Rousettus leschenaulti*; n=30			
	Number NB Virus Seropositive	**Number NB Virus Seronegative**	**Total**
Number Female	7	7	**14**
Number Male	3	13	**16**
Total	**10**	**20**	**30**
P Value	0.122		
Odds Ratio	4.3		
95% Confidence Interval	0.8, 22.2		

Conversely, Nipah virus antibody was more likely to be found in the *P. giganteus* males than the females, and this distribution difference was statistically significant (P=0.023) by Person's chi-square analysis. Sixty percent (60%) of the 42 *P. giganteus* males were antibody positive for Nipah virus whereas only 40% of the 48 *P. giganteus* females were positive, as displayed in Table 9. Explained in terms of the odds ratio, there was a 2.7 times greater odds of the Nipah virus seropositive *P. giganteus* bats being male as opposed to female and the odds were statistically significant based on the 95% confidence interval of 1.1-6.3.

Table 9. Nipah Virus Seroprevalence by Gender in the *Pteropus giganteus* Bats. The bats were collected during the 2004 human Nipah virus outbreak investigation in Bangladesh. Differences in the distribution by gender were statistically significant.

Species: *Pteropus giganteus*; n=90			
	Number Nipah Virus Seropositive	**Number Nipah Virus Seronegative**	**Total**
Number Male	24	18	**42**
Number Female	16	32	**48**
Total	**40**	**50**	**90**
P Value	0.023		
Odds Ratio	2.7		
95% Confidence Interval	1.1, 6.3		

Nipah and Nelson Bay (NB) Virus Seroprevalence Pattern Similarities with Respect to Mean Bat Body Measurements:

Table 10 gives the mean total body lengths, foot lengths, ear lengths, forearm lengths, and weights of the *R. leschenaulti* and *P. giganteus* bats in the 2004 Bangladesh bat cohort.

Table 10. *R. leschenaulti* and *P. giganteus* Mean Body Measurements. The mean total body lengths, foot lengths, ear lengths, forearm lengths, and weights of the *Rousettus leschenaulti* and *Pteropus giganteus* bats collected during the 2004 human Nipah virus outbreak investigation in Bangladesh.

	Species			
	Rousettus leschenaulti		***Pteropus giganteus***	
	Males	**Females**	**Males**	**Females**
Number Collected	16	14	42	48
Mean Total Body Length (mm)	120.9	119.0	236.1	237.6
Mean Foot Length (mm)	20.0	21.1	43.9	44.3
Mean Ear Length (mm)	20.3	20.3	36.4	37.2
Mean Forearm Length (mm)	84.6	82.8	170.8	167.2
Mean Weight (g)	107.6	96.2	678.6	621.9

No statistically significant difference in mean foot length or total body length was detected between the Nipah and NB virus antibody positive versus negative *P. giganteus* and *R. leschenaulti* bats per sex by independent T-test. There was, however, a statistically significant (at the $P \leq 0.10$ level) greater mean weight ($P=0.079$) and mean ear length ($P=0.061$) in the Nipah virus seropositive *P. giganteus* males as compared to the seronegative males. The 95 percent confidence interval around the difference in mean value was also calculated. This data is shown in Table 11. Note that all the mean body measurements were greater in the Nipah virus seropositve *P. giganteus* males compared to the seronegative males, although only the difference in mean ear length and mean weight was statistically significant.

Table 11. Independent T-test of Body Measurements in Nipah Virus Antibody Positive *P. giganteus* Males. Statistically significant values are marked with asterisks.

***Pteropus giganteus* Males: n=42**					
	Nipah Virus Antibody Positive	**Nipah Virus Antibody Negative**	**P Value**	**Difference in Means**	**95% Confidence Interval**
Mean Forearm Length (mm)	173.6	166.6	0.162	7.0	-2.9, 17.0
Mean Weight (g)	715.5	629.4	0.079*	86.0	-10.3, 182.3*
Mean Total Body Length (mm)	240.2	230.6	0.242	9.6	-6.8, 26.1
Mean Ear Length (mm)	37.5	34.9	0.061*	2.6	-0.1, 5.3
Mean Foot Length (mm)	44.4	43.2	0.628	1.3	-3.9, 6.4

Similarly, there was a statistically significant (at the P≤0.10 level) greater mean forearm length among the NB virus antibody positive *R. leschenaulti* females versus the seronegative females by independent T-test, as displayed in Table 16 below. Again, note that all the mean body measurements were greater for the NB virus seropositive *R. leschenaulti* females than the seronegative females, although only the difference in mean forearm length was statistically significant at the P≤0.10 level.

Table 12. Independent T-test of Body Measurements in NB Virus Antibody Positive *R. leschenaulti* Females. Statistically significant values (at the P≤0.10 level) are marked with asterisks.

***Rousettus leschenaulti* Females: n=14**					
	NB Virus Antibody Positive	**NB Virus Antibody Negative**	**P Value**	**Difference in Means**	**95% Confidence Interval**
Mean Forearm Length (mm)	84.1	81.4	0.067*	2.7	-5.7, 0.2
Mean Weight (g)	98.6	93.9	0.210	4.7	-12.5, 3.0
Mean Total Body Length (mm)	120.7	117.3	0.448	3.4	-12.9, 6.1
Mean Ear Length (mm)	20.9	19.7	0.351	1.2	-3.7, 1.4
Mean Foot Length (mm)	21.3	21.0	0.856	0.3	-3.6, 3.1

Nipah and Nelson Bay (NB) Virus Seroprevalence Pattern with Respect to Bat Roost Location:

The bats specimens obtained during the 2004 Bangladesh human Nipah virus outbreak investigation were collected from bats captured from 11 bat roosts found near the human settlements that experienced cases of Nipah virus infection. Each roost sampled contained approximately 100-200 individuals and roosts were located approximately 0.5 to 30 km from the areas where human cases were reported (Carroll et al., Publication in Preparation). The distribution of NB virus and Nipah virus seropositive bats by roost GPS location and species is shown in Table 13. Roosts 1, 2, and 11 contained both NB virus and Nipah virus seropositive individuals. All 11 roosts were Nipah virus antibody positive except for roosts 5 and 9. All 30 *R. leschenaulti* species bats were collected from a single bat roost (roost 1), ten of which (33.3%) were NB virus antibody positive. A map showing the locations of the roosts is presented in Figure 8. As indicated by the scale on the roost map, the greatest distance "as the bat flies" between any two roosts was approximately 76 km.

Table 13. Nipah and NB virus Seroprevalence by Bat Roost. Seroprevalence distributed by bat roost and species among the bats in the Bangladesh 2004 cohort. Bat roosts were located 0.5 to 30 km from the areas where human Nipah virus cases were reported. Note that roosts 1, 2, and 11 are NB and Nipah virus seropositive while all other roosts except 5 and 9 are seropositive for Nipah virus. There was not a statistically significant difference in the distribution of Nipah or NB virus positive males versus females within each roost per species (data not shown).

Bat Roost	Species	Number Collected	Percent Nipah Virus Antibody Positive	Percent NB Virus Antibody Positive
1	*Rousettus leschenaulti*	30	3.3%	33.3%
	Pteropus giganteus	1	-	-
2	*Pteropus giganteus*	15	33.3%	6.7%
3	*Pteropus giganteus*	6	66.7%	-
4	*Pteropus giganteus*	17	52.9%	-
5	*Pteropus giganteus*	1	-	-
6	*Pteropus giganteus*	12	50.0%	-
7	*Pteropus giganteus*	9	33.3%	-
8	*Pteropus giganteus*	14	50.0%	-
9	*Cynopterus sphinx*	2	-	-
	Megaderma species unknown	1	-	-
	Hipposideros species unknown	1	-	-
	Eonycteris spelaea	1	-	-
	Cynopterus species unknown	1	-	-
10	*Pteropus giganteus*	4	25.0%	-
11	*Pteropus giganteus*	11	45.4%	9.1%
	Total	**126**	**32.5%**	**9.5%**

Figure 8. Map of Bat Roost Locations. Map B (below) is an enlargement of the area in Central Bangladesh near Faridpur shown in red in Map A (at left). Map B depicts the locations of the 11 bat roosts where the 2004 Bangladesh cohort of bats were collected. The scale of the map is in the lower left corner. Roost locations are marked with red crosses. Nipah virus seropositive roosts (roosts 1-4, 6-8, 10-11) are indicated in white. Nipah and NB virus seropositive roosts (roosts 1, 2, and 11) are indicated in yellow. Map A is available at www.infoplease.com. Map B was created using Google Earth.

Virus Isolation Results:

Overall, 29.3% of the 168 bats from five species collected during the 2004 Bangladesh human Nipah virus outbreak investigation showed antibody evidence of Nipah virus infection by serology, but Nipah virus was not successfully isolated from any of the bat tissues (Carroll et al., Publication in Preparation). The virus isolation attempts made on the 168 bat spleens collected from the bats in the 2004 Bangladesh cohort yielded two unknown syncytia-forming (cell fusing) virus isolates. The two unknown isolates were obtained from the spleens of two *P. giganteus* bats: one from a male bat from roost 11 and the other from a female from roost 7 (See Table 13 and Figure 8). Roost 11 contained bats positive for both Nipah and NB viruses while roost 7 contained only Nipah virus antibody positive individuals.

Although the unknown isolates were initially believed to be paramyxoviruses, such as Nipah or Hendra viruses, electron microscopy by C.M. Goldsmith in the Infectious Disease Pathology Branch, CDC indicated that the virus isolates were orthoreoviruses. Similar to NB virus, the isolates produced unique fusogenic cytopathic effects in cell culture. However, the unknown isolates did not react with NB viral antibody by indirect immunoflourescent microscopic examination.

The unknown isolates displayed the growth pattern and the ultrastructural morphology of bat orthoreoviruses (which include NB virus, Pulau virus, and purportedly Broome virus). However, unlike the previously characterized bat orthoreoviruses (NB virus and Pulau virus), the isolates did not cross-react with NB viral antibody (Pritchard et al., 2006). The isolates are therefore distinct from Pulau virus and NB virus, the only other characterized bat orthoreoviruses, identified previously in 2005 and 1970,

respectively. Serologic assays developed from antigen extracts of the two unknown viral isolates did not detect antibody capable of recognizing the unknown viruses in the Bangladesh bat or Bangladesh human sera collections.

Chapter V: Discussion and Conclusion

The Implications of the Virus Isolation Results:

In a timely bat virology review article published March 2007 in *Clinical Infectious Diseases*, Halpin et al. presented the following figure showing the origins of the three previously identified bat orthoreoviruses (NB virus, Pulau virus, and Broome virus) along with the geographic distribution of *Pteropus* bats in Southeast Asia and Australia (Halpin et al., 2007). Broome virus, which has not yet been characterized in the literature, was purportedly isolated from a bat in Australia, as was NB virus, while Pulau virus was isolated from a bat in Malaysia.

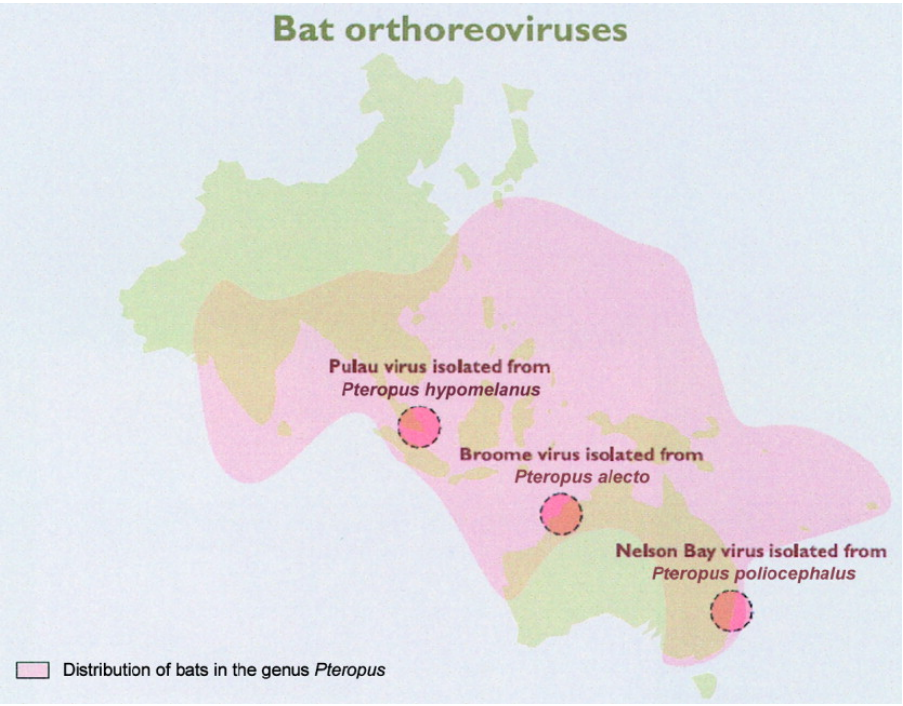

Figure 9. Map of the Bat Orthoreovirus Isolates. The geographic origins of the previously reported bat orthoreoviruses superimposed over the distribution of the *Pteropus* bat genus (pink shading) (Halpin et al., 2007, Fig 2, p. 714, *Clinical Infectious Diseases*).

This research reported the identification of two previously unknown bat orthoreoviruses obtained from the spleen tissues of the 168 bats in the 2004 Bangladesh cohort. The results of this research support hypothesis (1) which stated that culturing the bat tissues collected during the 2004 Bangladesh Nipah virus outbreak will result in the identification of novel viral agents. Therefore, Figure 9 could be updated with a fourth marker indicating the existence of both NB virus and a NB-like orthoreovirus in Bangladesh in addition to the three Australian and Malaysian bat orthoreoviruses the figure illustrates. Such an update to Figure 9 would further emphasize the broad distribution of viruses maintained in *Pteropus* bat reservoirs.

Future virus isolation studies will likely reveal the discovery of other bat orthoreoviruses in addition to the two new NB-like strains reported here. Bat viruses such as rabies, SARS, and Nipah viruses have often caused devastating disease in humans. Bat viruses from ancient viral families often have the ability to infect diverse groups of mammalian species since many use cellular receptors and metabolic pathways that were evolutionarily conserved in mammals as species diverged. The identification of novel bat viruses, even if they are nonpathogenic to humans, is of public health importance since it may help bat virologists further understand what characteristics separate the bat viruses that cause human disease from the greater pool of bat viruses that do not. Identifying the characteristics that enable bat viruses to cause human disease may allow public health officials to formulate intervention strategies to prevent or mitigate future outbreaks of bat viral disease.

Interpretation of the Nipah Virus Seroprevalence in the Human Specimen Catalog:

The human catalog screened consisted of 1,861 CSF, urine and serum specimens collected from India, Bangladesh, Thailand, and Indonesia in the years 2001 through 2006. The Special Pathogens Branch previously tested all specimens for Nipah viral antibody. None of the 73 urine specimens were positive for Nipah viral antibody. Of the 61 CSF specimens tested, only one specimen collected during the Bangladesh 2004 Nipah virus outbreak was Nipah virus antibody positive. Forty-six (46) of the 1,727 human serum specimens were Nipah virus antibody positive. As was displayed in Table 5, 82.6% of the 46 total Nipah virus antibody positive human sera specimens in the collection were obtained from the Nipah virus outbreak investigations conducted in Bangladesh in the years 2003, 2004, and 2005. Since the Bangladesh specimens made up 81.1% of the entire human sera catalog of 1,727 specimens, the specimens from Bangladesh were not over-represented among the 46 Nipah virus antibody positive specimens.

Although many of the Nipah virus seropositive human subjects in the screening were likely infected with Nipah virus via person-to-person transmission, the prominent modes of transmission varied widely in the previous human Nipah virus outbreaks described in the literature review chapter. Rather than from person-to-person transmission, some human cases among the Nipah virus outbreak patients screened became infected instead from direct exposure to the *Pteropus* bat reservoir of Nipah virus. Nipah virus is passed to humans from *Pteropus* bats through contact with environments contaminated with infectious bat secretions or excretions, through direct contact with the bats themselves (bats are hunted, killed, and prepared for food in some

Southeast Asian cultures), or through contact with an infected animal intermediary. The case-control studies that investigated the Bangladesh Nipah virus outbreaks indicated that regional behaviors associated with increased risk of Nipah virus infection included climbing trees near bat roosts or bat foraging sites, drinking a date palm sap beverage, and maintaining agricultural zones near bat habitats, as was summarized in Table 1.

In addition to Nipah virus transmission, these behaviors may put subjects at risk of contracting other viruses maintained in *Pteropus* bat reservoirs. *Pteropus* bats in the region are the suspected reservoirs of bat orthoreoviruses (such as NB virus) and other viruses posing a potentially greater disease risk to humans than orthoreoviruses, such as the newly identified lyssaviruses in Australia (similar to rabies virus) or the paramyxoviruses Tioman, Menangle, and Hendra viruses.

Interpretation of the Nipah Virus Seroprevalence in the Bat Sera Catalog:

The catalog of bat sera screened included 2,323 specimens from 27 rare bat species indigenous to Southeast Asia. *Pteropus* bats are the suspected primary bat reservoir of Nipah virus, although non-*Pteropus* species are also capable of becoming infected (Luby et al., 2006). Previous Nipah virus bat serosurveys have reported antibody evidence of Nipah infection in the following species of Megachiropteran bats (all within the Pteropodidae family): *P. conspicillatus, P. alecto, P. scapulatus, P. poliocephalus, P. hypomelanus, P. vampyrus, P. giganteus, P. lylei, Eonycteris spelaea, Cynopterus brachyotis, Scotophilus kuhlii, and Hipposideros larvatus* (Chua, Koh et al., 2002; Hsu et al., 2004; Olson et al., 2002).

As was determined by previous testing by Special Pathogens Branch microbiologists, Nipah viral antibody was present in the following seven bat species in the bat sera catalog: *P. giganteus*, *P. vampyrus*, *P. rodricensis*, *P. pumilus*, *P. lylei*, *P. hypomelanus*, and *R. lechenaulti.* Except for the *P. rodricensis, P. pumilus,* and *R. lechenaulti* species, all species in the catalog with Nipah viral antibody were previously reported in the literature as Nipah viral antibody carriers. The pie chart in Figure 5 showed that 67% of the 200 Nipah virus antibody positive bats were from the *P. giganteus* and *P. vampyrus* species, though these two species made up only 30.8% of the entire collection. This fact is not surprising since these bats are suspected to be the primary reservoir of Nipah virus in Southeast Asia (Chua, Koh et al., 2002; Hsu et al., 2004; Olson et al., 2002).

As is highlighted in yellow in Table 8, note that even though the 168 specimens in the 2004 Bangladesh bat cohort made up only 7.2% of the entire bat catalog of 2,323 specimens, 24.5% of the 200 Nipah virus antibody positive bat specimens belonged to the cohort. Hence, the Bangladesh 2004 cohort of bat specimens was over-represented among the 200 Nipah virus antibody positive specimens identified. The 2004 Bangladesh bat specimens were collected as part of the ecological serosurveys for Nipah virus in bats conducted during the human Nipah virus outbreak investigation in Bangladesh in 2004 (Carroll et al., Publication in Preparation).

There was a greater percentage (29.2%) of Nipah seropositive bats collected from Bangladesh in 2004 compared to all other bat specimen groups in the collection. In comparison, only 4.5% of the 44 *P. giganteus* bats collected in 2003 from Bangladesh were Nipah seropositive where as 44.0% of the 109 *P. giganteus* from Bangladesh in

2004 were seropositive (Carroll et al., Publication in Preparation). *P. giganteus* is the suspected primary reservoir of Nipah virus in Bangladesh. There was no difference in the percent of *P. giganteus* bats collected in Bangladesh in 2003 versus 2004 (78.6% of the bats collected 2003 were *P. giganteus* vs. 64.8% in 2004) (T.G. Ksiazek, Personal Communication). Therefore, the difference in Nipah virus antibody prevalence between the 2003 and 2004 Bangladesh bat cohorts may be due to factors intrinsic to the collection time, location, or sampling method rather than the species composition of the bats collected.

Nipah virus might naturally be more endemic to the area of Bangladesh where the 2004 cohort of bats were collected due to ecological or geographical factors. In addition, the bats collected in 2004 cohort may have been more strongly associated with a recent Nipah virus spillover event into humans resulting from increased Nipah virus prevalence in these bats. Finally, perhaps the timing of specimen collection was related to the increase in Nipah virus seroprevalence since the 2004 bats were collected from February through May of 2004 while the 2003 bats were all collected in March. The difference in Nipah seroprevalence may be explained by a multitude of factors impossible to tease out from the data at hand, none of which are mutually exclusive, or by some yet unappreciated determinant.

Implications of the Nelson Bay (NB) Virus EIA Results:

No diagnostic test for the presence of NB virus existed prior to this research. A successful EIA was developed to detect NB viral antibody in bats and humans. Subsequent serum neutralization testing confirmed that the NB virus antibody positive

specimens detected by the EIA contained antibody capable of neutralizing NB virus. The anti-bat conjugate employed in the NB virus EIA represents the first use of a conjugate specifically designed to recognize bats in both the Megachiropteran and Microchiropteran suborders. This conjugate is more sensitive and specific than the conjugate previously used in bat serosurveys that merely recognized the protein A and protein G immunoglobulin components generic to all mammals (J.B. Oliver, Publication in Preparation). This EIA may be a useful tool for public health professionals to detect disease if NB virus, or a closely related virus, spills out of the bat reservoir to cause illness in humans, domestic animals, or wildlife.

This research also represents the first reported attempt to study the prevalence of NB virus in wildlife or humans. No human specimens were NB virus antibody positive in the specimen catalog screened. NB virus antibody was successfully detected in 14 bats (0.6%) of three species (*R. leschenaulti*, *P. giganteus*, and *P. vampyrus*) out of the 2,323 specimens screened. Thirteen (13) of the 14 NB virus antibody positive bats were *Rousettus* and *P. giganteus* species and all 13 belonged to the cohort of 168 bat specimens collected during the 2004 Bangladesh human Nipah virus outbreak investigation. The only *R. leschenaulti* specimens in the catalog screened were also collected during the 2004 Bangladesh Nipah virus human outbreak investigation. Ten (10) of the 30 (33.3%) *R. leschenaulti* bats were NB virus antibody positive, as was displayed in Table 7.

Interpretation of the Nipah and Nelson Bay (NB) Virus Seroprevalence Pattern Similarities:

No human specimens were NB virus antibody positive, so similarities in the Nipah and NB virus seroprevalence patterns of were not detected in the human specimen catalog. As was displayed in Table 7, NB virus was successfully detected in three bat species (*R. leschenaulti*, *P. giganteus*, and *P. vampyrus*) that also carried antibody to Nipah virus. Therefore, the results support hypothesis (2), which stated that screening the bat sera catalog with the newly developed NB virus EIA would result in the successful detection of NB viral antibody among bat species in the sera catalog with Nipah viral antibody. The greatest NB virus antibody prevalence was found among the *R. lechenaulti* bats while the greatest Nipah viral seroprevalence was found among the *P. giganteus* bats.

In the 2004 Bangladesh cohort of 168 bats, the overall antibody prevalence of Nipah virus was 29.2% whereas the seroprevalence of NB virus was 7.7%. One striking similarity emerges from comparing the Nipah and NB viral seroprevalence patterns in the bat catalog; the 2004 Bangladesh bat cohort had the highest seroprevalence of both NB and Nipah viruses. This fact is very interesting since researchers have suggested that environmental conditions may increase the prevalence of zoonotically transmitted viruses in bats. Furthermore, it is interesting that the greater prevalence of Nipah viral antibody in the 2004 Bangladesh cohort of bats versus the 2003 Bangladesh cohort was not due to differences in the species composition of the bats collected, but rather due to factors intrinsic to the to the collection time, location, or sampling method.

The high frequency of human Nipah virus outbreaks in Bangladesh and the increased seroprevalence of both Nipah and NB viruses in the 2004 Bangladesh bat cohort may provide further evidence of the existence of larger environmental pressures that may be affecting the prevalence of bat viruses in the region. Larger environmental factors may also be markers of unbalanced ecosystems. Therefore, similarities in the seroprevalence patterns of bat viruses may be markers of unbalanced ecosystems.

Unbalanced ecosystems may promote the zoonotic transmission of a range of pathogenic agents to humans from many wildlife sources (Alcamo et al., 2003). Environmental determinants increasing the prevalence of known viral pathogens in bats may similarly be affecting the prevalence levels of viruses yet to be discovered that represent an unknown disease risk to human and veterinary public health (van der Poel et al., 2006). Consequently, identifying areas with an increased prevalence of bat viruses may allow public health researchers to target surveillance efforts for human diseases to appropriate high-risk environments. Careful monitoring of bat viral prevalence rates may also allow public health professionals the opportunity to develop intervention strategies to prevent or mitigate human disease outbreaks of bat-transmitted viruses.

The increased seroprevalence of both Nipah and NB viruses in the 2004 Bangladesh cohort of bats is confounded by the fact that 10 of the 14 NB virus seropositive bats were from the *R. leschenaulti* species. The only bats from this species in the catalog screened were a part of the Bangladesh 2004 cohort. Nelson Bay (NB) virus may naturally be more prevalent in *Rousettus* bats than any other species. Therefore, it unfortunately cannot be ruled out that the increased seroprevalence of NB virus in the 2004 Bangladesh bat cohort was simply due to the presence of *Rousettus* bats

in the cohort rather than due to the convergence of enabling environmental factors. Other bat sera collections containing specimens from *R. leschenaulti* bats should be screened for NB virus antibody to discern if NB virus is more endemic in the *Rousettus* species or if environmental factors unique to the location where the Bangladesh 2004 cohort of bats were collected could be increasing the prevalence of bat viruses in the area.

Interpretation of the Nipah and Nelson Bay (NB) Virus Seroprevalence Results with Respect to Mean Measurements of Bat Body Size and Gender:

Of the 120 bats in the 2004 Bangladesh cohort for which gender and body measurement data was collected, there was a statistically significant greater mean weight (P=0.018) and mean forearm length (P=0.035) in the Nipah virus antibody positive *P. giganteus* males as compared to the seronegative males by independent T-test. Similarly, at the P≤ 0.10 level, there was a statistically significant greater mean forearm length (P=0.067) among the NB virus seropositive *R. leschenaulti* females as compared to the seronegative females. Furthermore, all five of the mean body measurements studied were greater among the Nipah virus seropositive *P. giganteus* males and the NB virus seropositive *R. leschenaulti* females than in the respective seronegative bats, although only the mean forearm length and mean weight was statistically significant due to small sample size. These body measurements are used as surrogate measures of age in bats. This data implies that Nipah virus antibody positive male *P. giganteus* bats were more likely older than the *P. giganteus* seronegative male bats and that NB virus antibody positive female *R. leschenaulti* bats were older than the antibody negative females. There was also statistically significant differences in the distribution of Nipah and NB antibody by gender within the *P. giganteus* and *R. leschenaulti* bats. The observed differences in

the distribution of bat Nipah and NB viral antibody by gender and by mean body measurement may imply that bat mating is a significant source of viral transmission among bats.

Researchers have previously suggested that mating may serve as an important source of Nipah virus transmission among bats ("WHO-Wkly Epi Record," 2004). Only older bats mate and males tend to mate with several females in the mating season (Elangovan et al., 2002). Bangladesh Nipah virus human outbreaks have been reported yearly since 2003 and all occurred from the months of January through May, which coincides with the Bangladesh bat-breeding season ("WHO-Wkly Epi Record," 2004). The bat specimens from the 2004 Bangladesh cohort were collected from February through May during the breeding season and 92% of the female *P. giganteus* bats were pregnant at the time of specimen collection (Carroll et al., Publication in Preparation). Furthermore, earlier Hendra virus studies in *Pteropus* bats showed an increase in viral shedding during bat gestation and parturition, and so *Pteropus* bats may similarly shed more Nipah virus into the environment during this time, resulting in the subsequent transmission of Nipah virus to human populations sharing overlapping habitats (Halpin et al., 2007; Williamson et al., 1998). Alternatively, the seasonal cycle of human Nipah virus outbreaks in Bangladesh may be associated with seasonal human agricultural practices such as the collection of date palm sap from trees, usually occurring from mid-December through February (Luby et al., 2006), the availability of a seasonal bat food source, or some yet unappreciated cyclic risk factor ("WHO-Wkly Epi Record," 2004).

Conversely, the most likely reason for the statistically significant observed increase in the distribution of both Nipah and NB virus seroprevalence among larger (and

inferentially older bats) may simply result from the naturally expected accumulation of antibody over time among older bat populations (T.G. Ksiazek, Personal Communication). At the least, since the antibody positive bats were older than the negative bats, the antibody detected is likely not merely the result of maternal antibody transmission, but rather from individual exposure to these viruses. If, on the other hand, residual maternal antibody were primarily responsible for the Nipah and NB virus seroprevalence patterns observed, the antibody positive bats would have smaller mean body sizes than the antibody negative bats. It would be difficult to control for the effects of age or other cofounders in field studies examining bat mating and Nipah virus transmission since age and the onset of mating behaviors are inextricably linked.

Interpretation of the Nipah and Nelson Bay (NB) Virus Seroprevalence Results with Respect to Bat Roost Location:

Forty-two (42) suspected human cases of Nipah virus infection occurred from January-February of 2004 in the Rajbari province near the town of Goalando, Bangladesh. The bat specimens in the Bangladesh 2004 cohort were collected near Goalando from February-May of 2004 immediately following the human outbreak investigation and case-control study. Among the 126 bats collected from the 11 bat roosts, 41 (32.5%) were Nipah virus seropositive while 12 (9.5%) were antibody positive for NB virus. Roosts positive for NB viral antibody were clustered near the Nipah viral antibody positive roosts. Moreover, NB virus antibody positive bats inhabited the same roosts as Nipah virus seropositive bats in three of the 11 bat roosts (27.2%) studied. Out of the 13 total NB virus seropositive bats identified in the 2004 Bangladesh cohort, four (30.8%) were simultaneously antibody positive for both Nipah and NB viruses. It can

therefore be concluded that NB virus was circulating in *Pteropus* and *Rousettus* Megachiropteran fruit bats in Bangladesh at the time of the human Nipah virus outbreak and that the habitats of the NB virus seropositive bats potentially overlapped with the human settlements containing Nipah virus outbreak patients.

The case-control study conducted in the 2004 Bangladesh Nipah virus outbreak in the town of Goalando by Montgomery et al. determined that environmental exposure to bats during activities such as tree climbing were significantly associated with Nipah virus infection (Montgomery et al., Publication in Preparation). This research has demonstrated that NB virus was also prevalent in the bats that were the likely source of the human Nipah virus infections in 2004. It can be assumed that humans encounter Nipah and NB virus positive bats in a manner that has resulted in the effective transmission of Nipah virus from bats to humans. It is unknown if NB virus is capable of infecting humans. The lack of antibody to NB virus in the human populations screened supports the assumption that NB virus has not jumped species to infect humans, despite the opportunity for this transmission to occur. The lack of human NB virus antibody in the human specimen catalog was consistent with hypothesis (3), which stated that the newly developed EIA would not detect NB viral antibody in the catalog of human specimens previously screened for Nipah viral antibody. It is important for public health officials to be aware of the range of viral agents capable of causing human disease to effectively investigate outbreaks of unknown etiologies. This research, which investigated if NB virus is capable of infecting humans, is therefore of public health importance.

Four of the 13 NB virus antibody positive bats from the Bangladesh 2004 cohort were also Nipah virus seropositive. Furthermore, since Nipah and NB virus antibody positive bats shared roosts 1, 2, and 11 (shown in Table 13 and Figure 8), the ecologic conditions affecting the prevalence of Nipah virus may also affect the spread of NB virus in bats. It may even be possible for these viruses to co-infect a single bat host simultaneously. The investigators of the 2004 Bangladesh human Nipah virus outbreak observed that most bat activity near human Nipah virus cases was limited to foraging for food rather than roosting and most of the human cases of Nipah virus infection occurred in sites where bats foraged rather than roosted (Carroll et al., Publication in Preparation). This may imply that bats transmit Nipah virus to humans while foraging for food. Bat ecologists ascertain that *Pteropus* bats will forage for cultivated fruit in human agricultural settlements when their habitats or food sources are threatened, thereby increasing opportunities for human-bat interaction and the transmission of bat viruses via saliva on partially eaten fruit or through the contamination of palm sap beverages (Carroll et al., Publication in Preparation; van der Poel et al., 2006; Weiss & McMichael, 2004). Researchers have suggested that human encroachment in once isolated bat foraging habitats contributed to the emergence of the seasonal outbreaks of Nipah viral encephalitis in Bangladesh. Other dynamic environmental factors might similarly be altering the ecology of NB virus in bats.

The Public Health Importance of the Results of this Research:

This research found several similarities in the seroprevalence of both Nipah and NB viruses in the cohort of bats collected from Bangladesh in 2004 near the human

settlement that experienced cases of Nipah virus. The greatest seroprevalence of both Nipah and NB viruses was found in the 2004 Bangladesh bat cohort. The analysis of the mean body measurements of the 2004 Bangladesh bat cohort indicated that both the Nipah and NB virus seropositive bats were older than the seronegative bats. Differences in the distribution of NB and Nipah viral antibody by bat gender were also detected. In context of the previous Hendra virus transmission studies showing increased viremia during bat pregnancy and parturition, this analysis of seroprevalence with respect to mean bat body measurements and gender may further support the theory that seasonal bat pregnancy is associated with Nipah virus (and possibly NB virus) transmission between bats and from bats to humans. The analysis of seroprevalence with respect to roost location of the 2004 Bangladesh bat cohort indicated that the NB virus seropositive bats were clustered near the Nipah virus seropositive bats. Combined with the seasonal phenomenon of the Nipah virus outbreaks in Bangladesh, the results generated by this research may point to a strong environmental determinant affecting the prevalence of both Nipah and NB viruses in Bangladeshi bats.

An increase in the prevalence of a zoonotically transmitted virus in the bat reservoir may represent an increased risk of transmission to humans, potentially leading to outbreaks of disease. Environmental factors increasing the prevalence of viruses in bat reservoirs may then be risk factors for the occurrence of human disease. In addition to the two novel orthoreovirus isolates identified by this research, future bat viral ecology research will likely discover other bat viruses. The environmental factors increasing the prevalence of the known bat viruses may be similarly affecting the pool of yet

unidentified bat viruses that pose an unknown disease risk to human and veterinary public health.

Environmental risk factors increasing the prevalence of bat viruses may also be markers of unbalanced ecosystems. Unbalanced ecosystems promote the transmission of a range of pathogenic agents to humans from many wildlife sources, in addition to bats. Understanding the role these risk factors play in promoting disease may allow public health officials the opportunity to intervene to prevent bat-transmitted viral illnesses. Identifying the environmental risk factors that mark unbalanced ecosystems may also allow public health officials to target disease surveillance strategies to appropriate high-risk environments. Therefore, identifying the environmental risk factors affecting the prevalence of bat viruses is of public health importance.

The Environmental Determinants of Bat Viral Prevalence:

Several researchers have previously explored the roles environmental determinants may play in promoting disease outbreaks of Nipah virus in Southeast Asia. As classically depicted by epidemiologic triangle, the role that disease agents, disease hosts, and the environment play in the emergence of infectious disease outbreaks is particularly well illustrated by the research exploring the environmental causes of the Malaysian Nipah virus outbreak. The El Niño event from 1997-98 in Malaysia was one of the strongest of the century (P. R. Epstein et al., 2003; Patz, 2002). Researchers have theorized that the Malaysian monsoon season was upset by this El Niño phenomenon resulting in widespread drought, devastating forest fires, and hazardous pollution at the time of the Malaysian Nipah virus outbreak (Chua, Chua et al., 2002; P. R. Epstein et al.,

2003; Torres-Velez & Brown, 2004). Some researchers believe that *Pteropus* fruit bats infected with Nipah virus were swept onto Malaysian pig farms encroaching on previously uninhabited land while fleeing forest fires fueled by the intense drought (Chua, Chua et al., 2002; Daszak et al., 2001; P. R. Epstein et al., 2003; Newman et al., 2005; Torres-Velez & Brown, 2004). Concomitantly, a change to modern husbandry practices, employing crowded, high-density facilities and the movement of pigs among farms, created a convergence of enabling factors that provided the selective pressure necessary for Nipah virus to adapt to productively infect humans (J. H. Epstein et al., 2006; Feldmann et al., 2002; Newman et al., 2005). Purportedly, the intensity, timing, and distribution of extreme weather events, such as the El Niño phenomenon, are changing due to global climate change. These changes could potentially increase the worldwide incidence of many human infectious diseases (P. R. Epstein, 2005; P. R. Epstein et al., 2003).

As was noted by Epstein, among others, in his 2001 review in *Microbes and Infection*, "[c]limate is a key determinant of health. Climate constrains the range of infectious diseases, while weather affects the timing and intensity of outbreaks" (P. R. Epstein, 2001, p. 747). In other words, warming trends enable the geographic spread of zoonotic and vector borne infections while extreme weather events spawn clusters of outbreaks (P. R. Epstein, 2001; Patz et al., 2004).

According to an editorial written by Epstein and colleagues in the 2003 issue of *Environmental Health Perspectives,*

> "[a]s the climate becomes more unstable, its role [in disease emergence] increases. Having underestimated the rate at which climate would change, we are only beginning to understand the responses of biological systems to [global] warming and the accompanying intensification of weather extremes" (P. R. Epstein et al., 2003, p. A506).

McMichael offered a stunning example of the effects of climate change on disease. He observed that WHO attributed around 6-7% of the world's malaria cases directly to climate change (McMichael, 2004). Moreover, Patz (Patz, 2002) recognized that scientists at the ICDDR Institute in Bangladesh collected 18 years of data that demonstrated a relationship between cholera outbreaks in Bangladesh and increases in the sea surface temperature driven by El Niño phenomena (Pascual et al., 2000). As was later reemphasized by Epstein (P. R. Epstein, 2001), the Intergovernmental Panel on Climate Change concluded in 2001 that the only means to explain the warming climate of close to 1°C over the 20th century is the heat-trapping role of continuing greenhouse gas emissions (Houghton & Intergovernmental Panel on Climate Change. Working Group I., 2001).

Since trends such as global climate change are expected to continue, scientific efforts to explore the effects of these environmental risk factors on the appearance of infectious diseases, such as Nipah virus, should be intensified. The Henipavirus Ecology Collaborative Research Group is a multinational group of scientists that are using *Pteropus* field and laboratory data to study Nipah and Hendra viral dynamics in the bat population. As described by Daszak et al. (Daszak et al., 2004), they are attempting to

elucidate the roles that climate, deforestation, and anthropogenic landscape change may play in virus transmission (*The Henipavirus Ecology Collaborative Research Group*).

Land use change for agricultural purposes was a common denominator in the Nipah virus outbreaks in Bangladesh as well as in Malaysia (Fields et al., 2005; Newman et al., 2005; van der Poel et al., 2006). In chapter 18 of *Fields Virology* entitled Emerging Viral Diseases, Peters reminds readers that satellite images show that humans have altered over half the earth's land surface (Fields et al., 2005). Driven by the needs of an increasing human population, global deforestation continues at a rate of nearly three percent each year, leading to natural habitat destruction, a loss of species biodiversity, and exposure to new pathogens for livestock, wildlife, and ultimately, humans (Daszak et al., 2000; McMichael, 2004). Peters further reiterates that animal extinction rates have increased 100 to 1,000 times in recent decades so that many remaining species are isolated in small enclaves of genetically homogeneous individuals. Such selection pressures may therefore increase the concentration of EID pathogens in remaining populations of animal species (Fields et al., 2005).

Patz et al. asserted in a 2004 issue of *Environmental Health Perspectives* that the main environmental changes of anthropogenic origin that increase disease emergence risk include "deforestation, road construction, agricultural encroachment, dam building, irrigation, wetland modification, and mining" (Patz et al., 2004, p. 1092). McMichael additionally included "local/regional weather abnormalities, intensified crop and animal production systems, urban sprawl, continued poor sanitation, and the pollution of coastal zones" in a version of this list published in his 2004 *Philosophical Transactions of the Royal Society* article (McMichael, 2004, p. 1054).

In summary, Nipah emergence in Southeast Asia may have in part resulted from alterations in the movement and population density of *Pteropus* bats, brought on by a combination of deforestation, drought, and wildfires stemming from global climate change (P. R. Epstein et al., 2003; Newman et al., 2005). Although these environmental risk factors are capable of affecting the seroprevalence of Nipah virus and possibly NB virus, other factors have certainly played a role in the seasonal detection of Nipah virus in Bangladesh. For example, surveillance efforts to detect human cases of Nipah viral infection have intensified in the area since the disease was first reported and more cases of Nipah viral encephalitis are likely being recognized. Since most disease results from a complex web of causative factors with biological, socio-behavioral, and environmental qualities, it is difficult and sometimes impractical to attribute disease burden to single risk component. If the frequency or severity of Nipah viral outbreaks is indeed increasing in Bangladesh, human risk factors, such as a changes in behaviors and diet, bat habitat encroachment, new agricultural practices, or changes in the immune status of the local population may also be underlying causes of emergence (van der Poel et al., 2006). Furthermore, alterations in the virulence or pathogenicity of Nipah virus itself may be contributing to the broader disease spectrum observed in recent outbreaks.

Limitations of the Study:

It is important to qualify this discussion with the caveat that the ecologic conditions that affect Nipah viral prevalence may not necessarily similarly affect NB viral prevalence in bats since NB virus was more seroprevalent in *Rousettus* bats while more *Pteropus* bats were Nipah virus antibody positive. Although classified in the same

taxonomic subfamily, Pteropodinae, *Rousettus* bats may not share the behavioral characteristics of *Pteropus* bats. For example, bats of the genus *Rousettus* are the only Megachiropteran bats to use vocal echolocation by producing tongue clicks and therefore may have drastically different foraging, roosting, and mating behaviors (Elangovan et al., 2002).

A second limitation of this study is that many of the bat and human specimens screened were collected during Nipah virus outbreak investigations rather than specifically for the purpose of obtaining active surveillance data on the prevalence of bat viruses. Many of the bat species studied are rare or endangered. If they were available, additional specimens from Southeast Asia not associated with outbreaks of Nipah virus should be examined to assess the background prevalence of bat viruses in human and bat populations.

Recommendations:

Only 2.7% of the 1,727 total human serum specimens screened were Nipah virus seropositive. As a follow up to this study, Southeast Asian human populations with higher Nipah virus seroprevalence levels, and hence a greater level of exposure to the reservoir of NB virus, should be screened for NB viral antibody to lend more evidence to the conclusion that NB virus lacks the biologic capability to infect humans. In the 2004 Bangladesh cohort of 168 bats, the overall antibody prevalence of Nipah virus was 29.2% whereas the seroprevalence of NB virus was 7.7%. An increase in the seroprevalence of a bat virus biologically capable of infecting humans may represent an increased risk of viral spillover into humans, potentially resulting in human disease (Calisher et al., 2006;

Daszak et al., 2000). If the Bangladesh bat seroprevalence of NB virus were to increase so that it approached the 2004 Nipah virus seroprevalence level, it would be important to determine if NB virus antibody could subsequently be detected in Bangladeshi human settlements near the antibody positive bats.

Other CDC researchers have used the bat sera catalog to screen for SARS, Ebola, and lyssaviruses since many of the bat species represented in the catalog are rare. It would be worth exploring if any statistically significant patterns in seroprevalence by year, location, sex, or body measurement were noted for these viruses as was detected for both NB and Nipah viruses in the specimens from Bangladesh in 2004.

Ideally, long-term bat surveillance studies in Southeast Asia would further elucidate the range of viral agents associated with fruit bats and the factors affecting the prevalence of bat viruses. However, active surveillance for viruses in rare bat species would prove difficult and unwarranted. To thoroughly analyze the ecological determinants of bat viral prevalence, such surveillance studies should be conducted following the framework outlined by the Henipavirus Ecology Collaborative Research Group (http://www.henipavirus.org/index.html) and the discipline of conservation medicine. Only through the collaborative efforts of bat ecologists, virologists, and infectious disease experts could such surveillance studies definitively identify the environmental risk factors affecting the prevalence of bat viruses.

Conclusion:

Given that bats have served as the reservoirs of devastating human illnesses, bat viral ecology is an important, yet often underappreciated area of disease research (Breed et al., 2005; Calisher et al., 2006; Dobson, 2005; Halpin et al., 2007; Torres-Velez & Brown, 2004; van der Poel et al., 2006). Perhaps other studies like this one will result in a greater understanding of bat viral diversity and the factors that affect the prevalence of viruses in bats. Such efforts should help researchers identify the factors that distinguish the bat viruses that cause disease in humans from the greater pool of bat viruses that do not. It is likely that there are undiscovered bat viruses with the potential to cause human illness since many of the current bat-transmitted diseases are caused by viruses that belong to much larger ancient viral families thought to have evolved along with their bat hosts (Halpin et al., 2007; S. Wong et al., 2006).

As noted by Marano et al. in *Emerging Infectious Diseases*, "episodes of emerging zoonoses are being increasingly recognized around the world" (Marano et al., 2006, p. 1813). Marano et al. substantiate this claim with data compiled by Cowen et al. (Cowen et al., 2006) that "from 1996 to 2004, some 21% of 10,490 reports of animal diseases from 191 countries submitted to the Program for Monitoring Emerging Infectious Diseases (ProMED) concerned humans affected by zoonotic disease" (Marano et al., 2006, p. 1813). West Nile virus, SARS, monkeypox, and H5N1 avian influenza have shown the importance of working closely with the veterinary health profession to effectively detect and respond to emerging zoonoses (Chomel & Osburn, 2006; Torres-Velez & Brown, 2004). Instead of simply focusing on the next big health

crisis as it arises, researchers must endeavor to look upstream from specific disease risk factors to the underlying causes of disease emergence. Through ecological studies and overall species susceptibility investigations, scientists may explore the common denominators of disease emergence. Such research may lead to the identification of opportunities for intervention in the transmission of zoonotic diseases to humans (Torres-Velez & Brown, 2004; Weinhold, 2003)

Although zoonotic EIDs may not be a leading category of illness in the U.S., their perceived exotic nature and high mortality are great sources of concern (McMichael, 2004). Exotic zoonotic agents like Nipah virus may be a threat to our public health because of their risk to the agricultural industry, threat to the economy, and potential bioterrorism use (Kruse et al., 2004). Many foreign public health agencies have limited case management expertise and lack the appropriate resources to safely work with these zoonotic agents. Yet in today's global environment, one country unable to carry out early detection and response to animal disease outbreaks represents a liability to many other countries (Farmer, 1996; Zinsstag et al., 2007). To effectively respond to a potential event, the U.S. public health system must renew efforts to support the sophisticated infrastructure and trained personnel needed to respond to the spread of an EID outbreak (Fauci, 2001; Lederberg et al., 1992). Many of the social and environmental risk factors that contribute to EID spread are outside the traditional focus of the public health sector. To respond to the spread of EIDs there will be an increased demand for microbiologists, veterinarians, ecologists, epidemiologists, and modelers to work together in multidisciplinary teams (Chomel & Osburn, 2006; Davis et al., 2001; Farmer, 1996; Newman et al., 2005; Weinhold, 2003). Therefore, I believe more emphasis should be

placed on the ecological and social aspects of infectious disease emergence in training programs for public health professionals following the model outlined by the discipline of conservation medicine (McMichael, 2004; Weinhold, 2003).

In the modern global landscape, the U.S. public health system must be prepared to respond to new incidents of global infectious disease to meet the economic, humanitarian, and security demands of all people (Fauci, 2005; Lederberg et al., 1992). As Zinsstag et al. concluded in the May 2007 issue of *Emerging Infectious Diseases*, "[w]hen one considers health from a point of view independent of species, including humans, domestic animals, and wildlife, zoonoses are part of a broader ecologic concept of health systems" (Zinsstag et al., 2007, p. 527). I believe adopting this ecologic viewpoint of health will be a critical asset for public health professionals working to control the spread of EIDs in the future.

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